Mechanisms of glioblastoma cell migration and invasion: insights from the use of tyrosine kinase inhibitors

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I, Antonina Frolov, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Imatinib was the first targeted tyrosine kinase inhibitor (TKI) to be approved for clinical use, and remains first-line therapy for Philadelphia chromosome (Ph+)-positive chronic myelogenous leukaemia (CML). The second-generation inhibitor, nilotinib, was subsequently approved for treatment of CML patients that are resistant, or develop resistance, to imatinib. Imatinib was subsequently approved as front-line therapy for the treatment of gastrointestinal stromal tumours (GISTs), but failed clinical trials for glioblastoma multiforme (GBM). The present study shows that treatment of cultured glioma cells and glioma cells isolated from human biopsies with imatinib or nilotinib strikingly increases tyrosine phosphorylation of p130Cas, focal adhesion kinase (FAK) and paxillin (PXN), resulting in enhanced cell migration and three dimensional radial invasion. Imatinib and nilotinib-induced tyrosine phosphorylation and invasion is dependent on expression of p130Cas and FAK activity, and is not dependent on known imatinib and nilotinib targets including ABL, ARG, platelet derived growth factor receptor (PDGFR) and the collagen receptor DDR1. Interestingly, findings implicate the serine / threonine phosphatase PP2A in mediating tyrosine phosphorylation of p130Cas, FAK and PXN. Inhibition of PP2A with okadaic acid significantly increased basal levels of tyrosine phosphorylation of the three proteins. Conversely, pharmacological activation of PP2A using FTY720 strongly inhibited imatinib and nilotinib stimulated tyrosine phosphorylation. Imatinib and nilotinib also stimulate tyrosine phosphorylation and radial invasion in GIST cells, a cancer for which imatinib is already approved. These TKIs also stimulate tyrosine phosphorylation of p130Cas, FAK and PXN in hepatocellular liver carcinoma cells, and in non-transformed primary cell lines. Interestingly, Imatinib and nilotinib treatment of GBM cells results in increased serine phosphorylation of p130Cas, FAK and PXN. Findings here indicate important and unforeseen adverse effects of imatinib and nilotinib treatment on key motility-related pathways, which could be a significant contributor to the lack of clinical efficacy in GBM and to the development of resistance.

Publications

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Abbreviations

ABC = ATP-binding cassette (drug transporter)

ABL = Abelson tyrosine kinase (also known as ABL1)

AML = acute myeloid leukaemia

ARG = Abl-related gene product (also known as ABL2)

BCR = breakpoint cluster region

BRAF = B-Raf proto-oncogene

c-Kit = stem cell growth factor receptor (also known as CD117, and encoded by KIT)

c-Met = hepatocyte growth factor receptor

CCyR = complete cytogenetic response

CML = chronic myeloid leukaemia

CRAF = Raf-1 proto-oncogene

Crk = C10 regulator of kinase

CSC = cancer stem cell

CSK = C-terminal Src kinase

DDR1 = discoidin domain receptor tyrosine kinase 1

DMEM = Dulbecco's Modified Eagle's Medium

DMSO = dimethyl sulphoxide

ECM = extracellular matrix

EDTA = ethylenediaminetetraacetic acid

EGF = epidermal growth factor

EGFR = epidermal growth factor receptor

ERK = mitogen-activated protein kinase 1 (also known as MAPK)

FAK = focal adhesion kinase

FBS = foetal bovine serum

GBM = glioblastoma

GFP = green fluorescent protein

GIST = gastrointestinal stromal tumours

GSC = glioma stem cell

HCASMC = human coronary artery smooth muscle cell

HCC = human hepatocellular carcinoma

HGF = hepatocyte growth factor

ITGB1 = integrin β1

ITGB3 = integrin β3

LPS = lipopolysaccharide

MET = c-MET proto-oncogene

NCK = non-catalytic region of tyrosine kinase adaptor protein

NRTK = non-receptor tyrosine kinase

OA = okadaic acid

PBS = phosphate buffered saline

PDGF = platelet-derived growth factor

PDGFRα = platelet-derived growth factor receptor, alpha polypeptide

PDGFRβ = platelet-derived growth factor receptor, beta polypeptide

PFA = paraformaldehyde

Ph+ = Philadelphia chromosome positive

PP2A = protein phosphatase 2A, serine/threonine

PTP = protein tyrosine phosphatase

PTPN1 = protein tyrosine phosphatase non-receptor type; also known as PTP1B

PTPN11 = protein tyrosine phosphatase non-receptor type 1; also known as SHP2

PTPN12 = protein tyrosine phosphatase non-receptor type 12; also known as PTPPEST

PTPN14 = protein tyrosine phosphatase non-receptor type 14; also known as PTP-Pez

PTPRA = receptor protein tyrosine phosphatase α

PXN = paxillin

RTK = receptor tyrosine kinase

SCF = stem-cell factor

SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis

SFK = Src family kinase

SFM = serum-free medium

SH2 = Src-homology 2 domain

SH3 = Src-homology 3 domain

siRNA = small interfering RNA

SVZ = subventricular zone

TK = tyrosine kinase

TKI = tyrosine kinase inhibitor

WHO = World Health Organization

1 Introduction

1.1 The tumour microenvironment

1.1.1 The hallmarks of cancer

In their seminal paper Hanahan and Weinberg proposed that tumour growth and metastasis occurs via the acquisition of the following distinct and complementary capabilities, to which they refer to as the "hallmarks of cancer": resisting cell death, up-regulated proliferative signalling, evasion of growth suppressors, limitless replicative potential, sustained angiogenesis, and the ability for invasion (**Fig. 1.1.1**). The acquisition of these capabilities is dependent on alterations in the genomes of neoplastic cells. Genome maintenance systems govern genomic integrity in healthy cells by successfully detecting and resolving DNA defects. When these surveillance systems become deregulated, an accelerated mutation rate enables evolving premalignant cells to rapidly accumulate favourable genotypes, analogous to Darwinian evolution, which underpin the hallmarks of cancer (Hanahan and Weinberg, 2000).

A decade later, the two authors published an updated list of tumour hallmarks based on the progresses that had been made in understanding tumour biology. The revised list of hallmarks includes two enabling characteristics that allow for the acquisition of the six functional capabilities published earlier: genome instability and tumour-promoting inflammation (**Fig. 1.1.1**). The collapse of genomic maintenance machinery and increased sensitivity to mutagenic agents leads to genomic instability and increased mutability. A higher rate of mutation enables those cells with mutant phenotypes that confer advantage to survive, grow and eventually dominate in a local tissue environment. Infiltrated immune

cells are detected in virtually all tumours at varying densities where they mimic inflammatory conditions that arise in non-neoplastic tissues. The presence of immune cells in tumours was initially regarded as a reflection of the immune system's attempt to eradicate tumour cells. It later became apparent that tumour inflammation induces a paradoxical effect of promoting tumourigenesis and enabling the acquisition of the functional hallmarks of cancer. In some cancers, inflammation is detected at the earliest stages of tumour pathogenesis where it contributes significantly to the progression of early neoplasias into cancer by supplying tumour-promoting molecules to the neoplasm such as growth factors, proangiogenic factors and survival factors that prevent cell death (Hanahan and Weinberg, 2011).

Included in the revised list are also two emerging characteristics that are increasingly being recognised as important hallmarks contributing to tumour pathogenesis: the tumour cells' ability to adjust energy metabolism towards a higher rate of glycolysis to meet the growing requirement for energy needed to sustain cell division and proliferation; and their ability to evade destruction by the immune system (**Fig. 1.1.1**) (Hanahan and Weinberg, 2011).

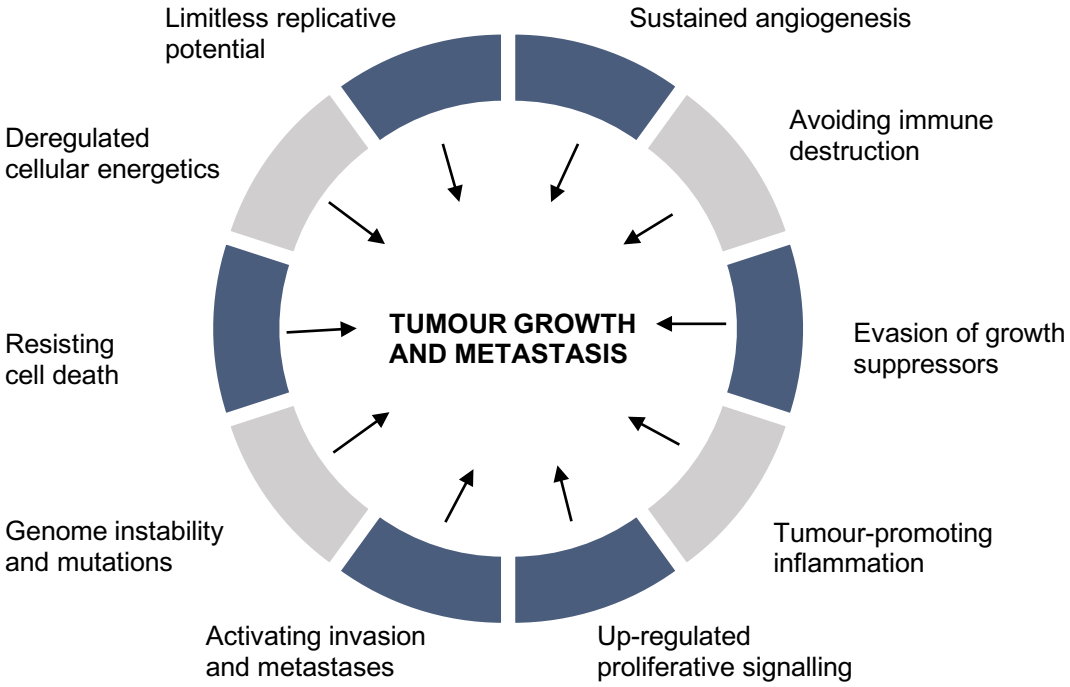


Figure 1.1.1 Hallmarks of cancer that contribute to tumour pathogenesis. The diagram includes the six original hallmarks proposed by Hanahan and Weinberg in 2000 which enable tumour growth and spread (in blue), and the additional four emerging hallmarks proposed in 2011 (in grey). *[Figure adapted from (Hanahan and Weinberg, 2011)]*

1.1.2 Tumour heterogeneity

Neoplasms originate from normal cells that accumulate genetic and epigenetic alterations required for transformation. Even though spontaneous tumours originate from a single cell, by the time of clinical diagnosis, most tumours display heterogeneity in their phenotypic features to include cellular morphology, gene expression, metabolism, motility, and angiogenic, proliferative, immunogenic, and metastatic potential (Marusyk and Polyak, 2010). Although the "hallmarks of cancer" which enable tumour progression are the same, the particularities driving growth differ according to environmental stresses that are present. Tumour heterogeneity is crucial to malignancy as it impedes diagnosis and poses great challenges to the design and choice of effective therapies. Tumours within a single organ differ, and this is referred to as *inter-tumour* heterogeneity. Importantly, cells within the same tumour also differ. The tumour microenvironment affecting tumour cells is not homogeneous, requiring phenotypic and genetic differences for cell survival. Differences in oxygen pressure, access to blood vasculature, and composition of the extracellular matrix can all be observed within a single tumour, all of which place different pressures on cells. The changing tumour microenvironment leads to the emergence of different clones within a single tumour, referred to as *intra-tumour* heterogeneity (Inda et al., 2014).

The existence of heterogeneity has long been recognised, but the relative contributions of different mechanisms is still not clear. The widely accepted Darwinian model to explain the progression of cancer stipulates that genetic or epigenetic mutations occur at random and favourable phenotypes produce clones best fit to survive environmental changes and these outgrow other clones. The genetic heterogeneity caused by changes in the genome translates into phenotypic heterogeneity. Within the framework of this model, heterogeneity could be explained by the presence of remaining weaker clones generated during tumour growth. In the context of chemo- or radiotherapy, acquiring resistance allows a population of cells to respond to a selection pressure, evade treatment, survive and expand (Inda et al., 2014, Marusyk and Polyak, 2010). Darwinian clonal evolution is supported by genome-wide studies and advances in next-generation sequencing. Many studies have identified genetic heterogeneity within individual cancers, revealing distinct clonal subpopulations that represent sequential clonal expansions. (Zellmer and Zhang, 2014, Navin et al., 2011).

1.1.3 Cancer stem cells

The view of heterogeneity based on principles of Darwinian evolution stipulates that a single somatic cell with a heritable mutation conferring survival advantage will proliferate and outlive other cells. Consequently, natural selection leads to clonal expansion, and cells within a tumour share common genetic abnormalities which can reflect their clonal origin. But single-cell analysis also shows differences in genetic and epigenetic abnormalities between individual cells. The major problem in cancer research is identifying the cell capable of initiating and sustaining growth of the neoplastic clone. More recently, the cancer stem cell (CSC) theory was proposed, stipulating that only a small fraction of tumour cells with the ability to self-renew and differentiate into "non-stem" cells are responsible for the maintenance of tumours (Marusyk and Polyak, 2010, Dick, 2008). Both the CSC theory and the clonal evolution model have been proposed to explain intra-tumour heterogeneity. The CSC theory proposes that a subset of cells with stem cell properties drive tumour initiation and progression. By contrast, the Darwinian clonal evolution model proposes that cells are subject to natural selection. A malignant cell accumulates hereditary changes over time that confer survival advantage that allow it to survive and expand (Michor and Polyak, 2010).

The term "cancer stem cell" refers to the distinct cell within the neoplastic clone that is capable of initiating and sustaining tumour growth *in vivo* (Wang and Dick, 2005). The CSC model stipulates that a small number of phenotypically distinct tumorigenic cells form the majority of non-tumorigenic and phenotypically diverse cells that comprise the cancer. The now widely accepted CSC model of cancer evolution and heterogeneity was postulated initially for hematopoietic malignancies. Research into acute myeloid leukaemia (AML) revealed that cells have limited proliferative capacity, which suggested the malignant clones are maintained by a rare population of stem cells (Sawyers et al., 1991). Pluripotent stem cells in the bone marrow had been established, expressing high levels of cell surface marker CD34 (Civin et al., 1984). The subsequent development of xenotransplantation protocols using immunodeficient mice enabled the *in vivo* detection of stem cells capable of initiating cancer. It was discovered that distinct leukaemic cells derived from the peripheral blood or bone marrow of AML patients could reconstitute the disease when transplanted into immunodeficient mice; whereas all other leukaemic cells could not. These cells were found to express CD34 and lack CD38. Expression of CD38 on CD34-positive cells is a marker

for lineage commitment, suggesting that the cells able to reconstitute AML in mice were immature bone marrow cells (Lapidot et al., 1994).

The ability to purify and analyse distinct CSCs has pointed to the presence of distinct heterogeneous types of stem cells with differing proliferative, differentiating, and self-renewal capacities. The presence of CSCs provides evidence that non-heritable sources of tumour heterogeneity exist, by contrast to the Darwinian model of clonal evolution that relies on acquired genetic and epigenetic mutations. Importantly, the two models are not mutually exclusive and both are relevant to cancer initiation and progression. Tumour cell plasticity is an additional non-heritable source of heterogeneity, defining the phenotypic differences that arise due to changes in tumour microenvironment. In contrast to the CSC model, it has been found that heterogeneity in melanoma is driven by reversible phenotypic changes *in vivo*, whereby both the melanoma cells expressing and lacking specific cell surface markers are able to form tumours, and that the tumours exhibit similar heterogeneity of marker expression (Quintana et al., 2010, Shackleton et al., 2009, Inda et al., 2014).

The interaction of tumour cells with their surrounding microenvironment shapes tumour progression and malignancy. Tumour cell plasticity enables cells to adapt to differences in the microenvironment by producing diverse phenotypes. Confounding diagnosis and treatment further, is the concept of inter-clonal cooperativity. This model stipulates that clones which possess a pro-oncogenic microenvironmental phenotype may alter the microenvironment through the secretion of extracellular factors to promote the growth of other clones (Inda et al., 2014).

Clonal heterogeneity remains a poorly explored phenomenon. The co-existence of diverse clones within a tumour leads to complicated signalling network between the clones and the microenvironment and amongst the clones. Tumours are treated as complex systems, the complexity of which confounds diagnosis and impedes treatment. A particularly malignant type of cancer is glioblastoma multiforme. This glioma exhibits relentless progression via aggressive invasion and resistance to therapy as a result of widespread heterogeneity.

1.1.4 Glioblastoma multiforme

Gliomas are glial malignancies, classified based on histopathological characteristics, according to World Health Organization (WHO) classification, into astrocytomas, oligodendrogliomas, or mixed oligoastrocytomas with morphological features of both astrocytes and oligodendrocytes. Tumours are then graded on a WHO scale of I-IV according to their level of malignancy (Furnari et al., 2007). Gliomas exhibit genetic differences as well as different cells-of-origin. However, they are similar in their ability to aggressively invade surrounding tissue (Cuddapah et al., 2014).

Primary brain tumours account for 7% of lives lost yearly from cancer before the age of 70 (Furnari et al., 2007). Glioblastoma multiforme (GBM) is a WHO Grade IV astrocytoma, and represents more than 15% of all primary brain tumours (Persano et al., 2013, Hou et al., 2006). GBMs are heterogeneous masses that are made up of glioma cells, hyper-proliferative endothelial cells, macrophages and trapped cells of the normal brain structures (Holland, 2001). GBM is the most aggressive malignant glioma, characterised by uncontrolled proliferation, diffuse infiltration, angiogenesis, resistance to apoptosis, and high genomic instability (Furnari et al., 2007).

An increasing amount of genetic alterations often correlates with increasing severity of clinical outcome. Genetic mutations largely fall into two distinct categories, altering either signal-transduction pathways downstream of tyrosine kinase receptors, or disrupting cell-cycle arrest pathways. The expression of growth factors and receptors that control glial cell differentiation are frequently elevated in gliomas, such as platelet-derived growth factor (PDGF), platelet-derived growth factor receptor (PDGFR), or epidermal growth factor receptor (EGFR) which is amplified in 30-50% of malignant gliomas. Excessive production of growth factor and growth factor receptors results in autocrine stimulation and increased downstream signalling (Holland, 2001). The p53 tumour suppressor represses abnormal cell proliferation and growth. It controls the arrest of the cell in the G_1 phase, initiates DNA repair, induces programmed cell death, and promotes cellular differentiation (Louis, 1994). The gene encoding p53, TP53, is mutated in approximately 40% of GBM (Holland, 2001).

More than 22,000 people a year are diagnosed with a malignant glioma in the USA alone. Therapy consists of a maximal tolerable surgical resection followed by radiation and

chemotherapy. However, this adds only several months of additional survival and the median patient survival time is only 12 to 18 months, with 95% of patients dying within 5 years. Treatment remains ineffective because of the high intrinsic ability of GBM to invade surrounding tissue and by strong inter- and intra-tumour heterogeneity. Tumour cells aggressively diffuse into surrounding brain area, and recurrence after surgical intervention is inevitable. Gliomas show excessive intra-organ invasion but, by contrast to other cancers such as small-cell lung carcinoma or prostate cancer, less than 2% metastasise beyond the brain. But the high intrinsic ability to invade neighbouring tissue and to infiltrate the brain parenchyma renders GBM highly resistant to traditional therapeutic approaches (Cuddapah et al., 2014, Hou et al., 2006).

A large effort has been made to understand GBM cell migration and invasion with a large number of studies being designed to delineate the molecular mechanisms underlying these processes. Development of clinically relevant tumour model systems for understanding GBM is essential for the advancement of basic biology and translational medicine. Numerous cell line models of GBM have been established which contain genetic abnormalities typical of primary cancers and provide the basis of significant biological insight into the disease (Clark et al., 2010).

1.1.5 Glioblastoma multiforme heterogeneity

Glioblastoma multiforme is characterised by high levels of intra-tumour heterogeneity, as the term multiforme suggests. After the brain develops, a population of stem cells persists in the subventricular zone (SVZ), the structure situated throughout the walls of the lateral ventricles. These stem cells proliferate and retain the capacity to generate neurons and glial cells following embryonic development. The SVZ stem cells have been identified as a source of a brain tumours and can give rise to tumours with diverse histological phenotypes. Deletion of the tumour suppressors p53 and retinoblastoma protein (Rb) in the mouse SVZ leads to the development of a malignant neural crest tumour. Deletion of p53 and PTEN in the mouse SVZ stem cells results in a tumour that resembles human anaplastic oligoastrocytoma, which is a tumour that arises from the rapid proliferation of oligodendrocytes and astrocytes. By contrast, mouse astrocytes with deletion of Rb/p53/PTEN, Rb/p53 or PTEN/p53 proliferated strongly *in vitro* but did not generate tumours *in vivo*, suggesting that the pathways that generate tumours from SVZ stem cells do not form tumours from astrocytes (Jacques et al., 2010). These findings support the role of stem cells in initiating malignancy, and suggest that the combination of stem cell genetic mutations underpins the specific phenotype of a tumour.

Glioma stem cells (GSCs) reside in specific tumour niches and are capable of generating new tumours when transplanted. They share properties to normal stem cells, are driven by microenvironmental factors such as hypoxia, and are able to initiate and maintain a malignant tumour. GSCs are responsible for tumour maintenance, enabling resistance to chemo- and radiotherapy, and are important in recurrence after resection (Persano et al., 2013).

GSCs were initially defined by the expression of the glycoprotein CD133 (prominin-1) (Singh et al., 2004). But CD133-negative cells are also able to form tumours in immunodeficient mice, suggesting the presence of other markers that need to be delineated (Inda et al., 2014). More recently, a population of glioma cells expressing high levels of CD44 and the DNA-binding protein-1 were identified that confer poor prognosis in GBM patients and display a stem-like phenotype (Anido et al., 2010).

Clusters of proliferating neural stem cells have been found to occupy a vascular niche in gliomas and contain proliferating endothelial precursors, suggesting a role for angiogenesis. In addition, endothelial cells secrete factors that can stimulate these stem cells to proliferate. It has been reported that subsets of glioma cells retain stem cell like properties and that retroviral delivery of oncogenes, such as PDGF, into neural progenitors is sufficient to induce a diffusely infiltrating tumour that mimics human glioma (Farin et al., 2006).

There is also evidence of GBM inter-clonal cooperativity. Simultaneous engraftment of cells over-expressing wildtype EGFR (wtEGFR) and mutated EGFRvIII resulted in GBM tumours that expressed wtEGFR in much larger quantity than the mutated receptor. The study revealed that a paracrine mechanism driven by EGFRvIII is the major cause for wtEGFR-expressing cell recruitment. The EGFRvIII-positive cells express specific cytokines, which activate wtEGFR in neighbouring cells. The deletion of these cytokines abolishes cross-talk between cells and significantly attenuates tumour growth (Inda et al., 2010).

1.2 Cell motility

The acquired ability for invasion and metastasis enables cancer cells to escape the primary tumour and occupy a new area in the body where nutrients and space are not limiting. The resulting distant settlements of tumour cells represent the cause of approximately 90% of human cancer-related deaths (Hanahan and Weinberg, 2011).

Invasion and metastasis accounts for the difference between benign and malignant tumours. Invasiveness refers to the combined active invasion and destruction of tissue. Metastasis refers to the capability to leave a primary tumour and form a secondary tumour (Leber and Efferth, 2009). Tumours metastasise in two ways: they spread to other organs, usually via the blood or the lymphatic system; and they metastasise locally to form new tumours within the same organ (Cuddapah et al., 2014).

Metastasis is caused by invasion and migration of individual cells, which detach from the primary tumour and invade surrounding healthy tissue. Malignant cells then attach to endothelial cells via adhesion molecules and secrete enzymes that allow them to infiltrate blood vessels and enter the blood circulation. Particularly aggressive and resistant cells will survive the toxic concentration of oxygen and lymphocytes. Tumour cells will get stuck in the capillaries of organs and will penetrate the endothelium of the organ by proliferating and utilizing proteolytic enzymes. Cells colonise the organ by proliferating and inducing angiogenesis, or they may coopt existing host blood vessels, to ensure vascularisation for survival. This cascade of events results in the formation of distant secondary tumours (Leber and Efferth, 2009).

Invasion and metastasis are complex processes, the precise biochemical and genetic determinants of which remain poorly understood. The pathways which govern healthy cellular motility become deregulated causing excessive migration, invasion and metastasis. Growth factor receptors are at the top of downstream signalling cascades that regulate fundamental cellular processes including migration, as well as proliferation, metabolism, differentiation, and programmed cell death. Cell migration depends on growth factor stimulation via binding to receptor tyrosine kinases (RTKs), integrin receptor activation, and

subsequent activation of downstream signalling molecules including non-receptor tyrosine kinases (NRTK), adaptor and scaffold proteins, and small GTPases (**Fig 1.2**).

Activation of RTKs leads to increased tyrosine phosphorylation of key signalling molecules required for motility. Among these are p130Cas, focal adhesion kinase (FAK) and paxillin (PXN). For example, p130Cas tyrosine phosphorylation is increased by PDGF, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF). Tyrosine phosphorylation of p130Cas plays a critical role in PDGF-induced migration of U87MG glioma cells and vascular smooth muscle cells (Barrett et al., 2014).

Focal adhesions are transient self-assembling complexes that enable the cell to make contact and interact with the extracellular matrix (ECM) and facilitate the connection between the ECM and actin cytoskeleton. Focal adhesion dynamics (assembly and disassembly) is a continuous process, and involves fibronectin, filamin and integrin association, that together with adaptor proteins such as p130Cas and PXN, and signalling proteins such as Src and FAK, structurally and functionally control the cell's morphology and are required for cell motility (**Fig.1.2**) (Campbell, 2008, Hu et al., 2014). NRTKs Src and FAK form a signalling complex in response to integrin or growth factor receptor stimulation. This complex promotes GTPase activation through the phosphorylation of two major adaptor proteins, PXN and p130Cas (Arregui et al., 2013). Knock-out mutations in Src or FAK, or adaptor proteins p130Cas or PXN, results in embryonic lethality attributed to the failure of cell migration (Sharma and Mayer, 2008).

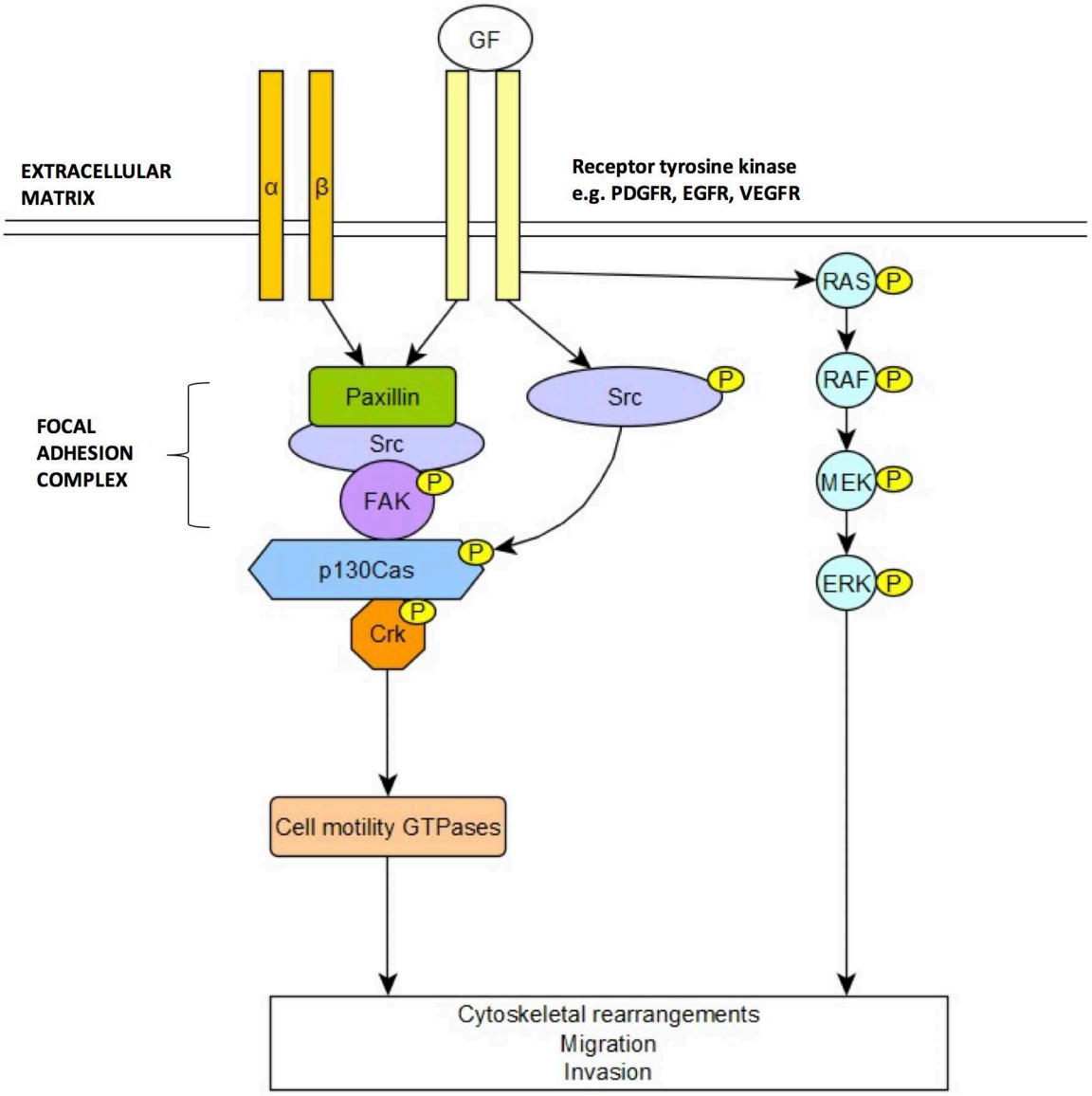


Figure 1.2 Key motility signal transduction pathways. p130Cas co-localises with proteins that make up focal adhesion complexes –paxillin, FAK, and Src. Paxillin, FAK, Src and p130Cas are activated via either integrin receptor or tyrosine kinase receptor stimulation. Receptor tyrosine kinases are activated by growth factor (GF) binding. Activated receptors phosphorylate FAK, which leads to recruitment and binding of Src. Activated receptor tyrosine kinases also phosphorylate Src, which then binds to a Src-binding site located in the carboxy-terminal domain of p130Cas and is responsible for the direct phosphorylation of p130Cas in response to numerous growth factors. Crk adaptor proteins link p130Cas to downstream effectors which catalyse the activity of small GTPases, bringing about changes in the actin cytoskeleton to facilitate cell motility. RAS is activated by receptor tyrosine kinases and recruits RAF to the membrane. RAF phosphorylates and activates MEK, which then phosphorylates and activates ERK (Barrett et al., 2013, Heidorn et al., 2010).

1.2.1 Focal adhesions

1.2.1.1 Integrins

Integrins are transmembrane receptors that enable cells to sense their environment. They are cell adhesion molecules implicated in cell motility that transmit signals bi-directionally across the plasma membrane to mediate cell–cell, cell–extracellular matrix (ECM) and cell–pathogen interactions (**Fig.1.2**) (Cuddapah et al., 2014). Integrin adhesion receptors span the cell membrane to link the ECM with intracellular signalling proteins. The cytoplasmic protein talin regulates integrin affinity and provides a mechanical link between integrins and the actin cytoskeleton. Each integrin subunit contains a large extracellular domain, a single transmembrane domain and a short cytoplasmic tail. 18 α subunits and 8 β subunits dimerise to form 24 different integrins that display distinct binding affinities for particular ligands. For example, integrins containing subunits α_7, β_1, and β_3 have been shown to induce p130Cas phosphorylation (Luo and Springer, 2006, Campbell, 2008, Ye et al., 2014).

Integrin receptor activation following adhesion to the ECM is considered the initial trigger to activate downstream signalling cascades mediated by non-receptor tyrosine kinases. Cell adhesion to ECM proteins such as fibronectin and vitronectin initiates integrin activation, which in turn leads to the FAK autophosphorylation at tyrosine residue Y397, which recruits Src and p130Cas (Sharma and Mayer, 2008). Integrin-mediated phosphorylation of p130Cas is Src-dependent and often results in the establishment of Cas-Crk complexes. The C10 regulator of kinase (Crk) is an adaptor protein with Src-homology 2 (SH2) and SH3 domains that couple to effectors p130Cas and PXN (Bouton et al., 2001, Barrett et al., 2013). The cooperative signalling between integrins and growth factor receptors accessing the intracellular signalling space modulates this complex and regulates downstream signalling involved in cell proliferation, differentiation and motility (Eke and Cordes, 2014).

Changes in integrin expression are evident in invasive and metastatic cells, with shifts in the spectrum of integrin α or β subunits displayed by the migrating cells (Hanahan and Weinberg, 2000). β1 integrin (ITGB1) is often up-regulated in human malignancies and is implicated in tumour progression and metastasis. For example, up-regulation of ITGB1 has recently been found to significantly induce proliferation and invasion of human colorectal cancer cells (Song et al., 2014).

1.2.1.2 Adaptor proteins

Adaptor proteins, also known as scaffold proteins, provide structural framework to facilitate the concurrent binding of proteins within particular signalling pathways. Adaptor proteins are composed of multiple protein-binding domains that mediate the association of various binding partners. By linking specific proteins together, cellular signals can be propagated to achieve a response to particular environmental cues. Specificity in signalling is achieved by the type of protein binding domains encoded by the adaptor protein, the sequence of domains that dictates specificity in binding, subcellular localisation and proximity of binding partners. Adaptor proteins are positioned to regulate cell signalling in a spatial and temporal manner and enable sequential activation of individual components of signalling pathways (Flynn, 2001).

1.2.1.2.1 Paxillin

Paxillin (PXN) is a 559 amino acid protein that localises to focal adhesions, the sites at which the cell makes contact and interacts with the ECM (**Fig.1.1**). Its primary function is to serve as a scaffold protein, providing multiple docking sites at the plasma membrane for signalling and structural proteins. PXN recruits various cytoskeletal and signalling proteins into a complex, including Src and FAK, which are activated as a result of adhesion or growth factor stimulation, thus enabling the coordination of downstream signalling that results in cell motility (Schaller, 2001, Turner, 2000b).

The N-terminal half of PXN contains five copies of an eight-amino-acid leucine-rich sequence known as the LD motifs (LD1-5), which serve as binding sites for proteins (**Fig. 1.2.1.2.1**). FAK binds PXN at LD2 and LD4. The focal adhesion-associated protein vinculin that functions to link the actin cytoskeleton to focal adhesions was the first PXN binding partner to be identified and associates with LD1, LD2 and LD4 (Schaller, 2001). The C-terminal half of paxillin is comprised of four LIM domains (LIM1-4), each of about 50 amino acids and arranged in tandem. LIM domains are double zinc-finger motifs that function mainly to anchor PXN at the plasma membrane. Phosphorylation on serine and threonine residues of PXN LIM2 and LIM3 domains during cell adhesion or growth-factor stimulation potentiates the localisation of paxillin to focal adhesions (Turner, 2000a).

PXN has been shown to be a cellular target for tyrosine kinases that are activated as a result of integrin and growth factor signalling. Phosphorylation of PXN is associated with the coordinate formation of focal adhesions and is important for their association with actin filaments. FAK, in association with Src that binds FAK at Y397, phosphorylates PXN at tyrosine residues Y31 and Y118. Phosphorylation at these sites generates two functional SH2-binding sites for members of the Crk family of SH2-SH3 adaptor proteins. Crk binds p130Cas following its phosphorylation by Src. The association of Crk with p130Cas and PXN is important in mediating cell motility (Turner, 2000a). Overexpression of paxillin mutants in bladder tumour cells in which tyrosine Y31 and/or tyrosine Y118 are replaced by phenylalanine prevents the formation of PXN-Crk complexes and impairs cell motility (Petit et al., 2000).

Negative regulators of signalling pathways involved in migration also bind PXN, including CSK (an inhibitor of Src activity) and PTP-PEST (a phosphatase that dephosphorylates p130Cas). By binding to PXN they are brought into close proximity with their targets (Turner, 2000b).

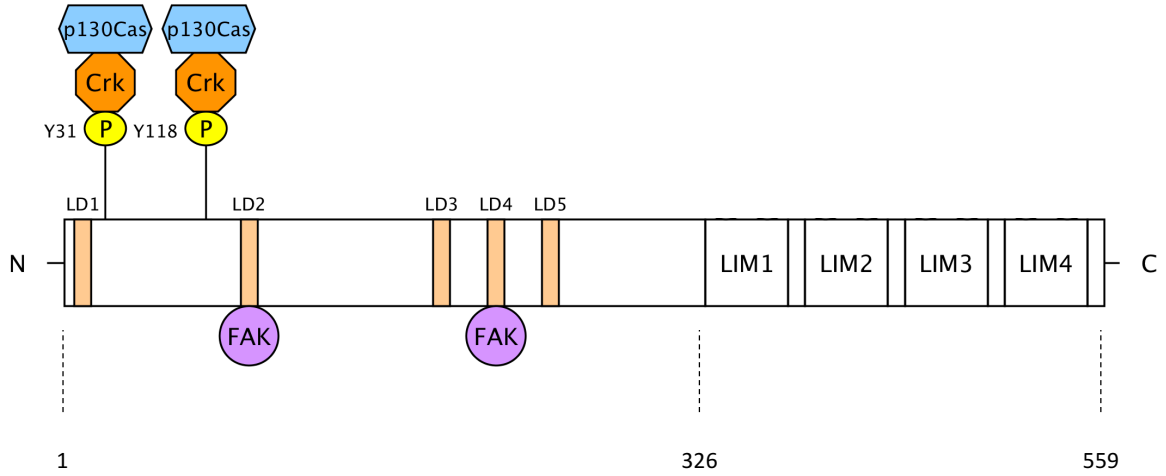


Figure 1.2.1.2.1 Structure of paxillin. The adaptor protein PXN serves as a scaffold for the recruitment of many signalling proteins to the plasma membrane. Human paxillin isoform 1 contains 559 amino acids and the amino acid sequence numbers are represented beneath the figure (Turner, 2000a). The N-terminal domain contains five eight-amino-acid leucine-rich sequences known as LD motifs (LD1-5), which function as binding sites for proteins. FAK binds to LD2 and LD4. The C-terminal half of PXN is made up of four LIM domains arranged in tandem, which are double zinc-finger motifs, each of about 50 amino acids, that function mainly to anchor PXN at the plasma membrane. FAK in association with Src phosphorylates (P) PXN at two main sites – tyrosine residues Y31 and Y118, generating two functional SH2-binding sites for members of the Crk family of SH2-SH3 adaptor proteins. This enables the formation of PXN-Crk complexes, important for linking p130Cas (Schaller, 2001, Turner, 2000a). *[Figure adapted from (Turner, 2000a)]*

1.2.1.2.2 p130Cas

p130cas (also known as BCAR1) is a member of the Cas (Crk-associated substrate) family of adaptor proteins, which includes NEDD9 and BCAR3. It is a 130 kDA protein which lacks a kinase domain, but contains various protein-protein interaction domains that mediate associations with a number of binding partners (**Fig 1.2.1.2.2**). p130Cas possesses an amino (N)-terminal SH3 domain, followed by a proline-rich region (PRR) and a substrate domain. FAK interacts and co-localises with p130Cas at focal adhesion complexes by binding to the p130Cas SH3 domain. The substrate domain is comprised of 15 repeats of the YxxP consensus phosphorylation motif for Src family kinases. These YxxP motifs create docking sites for SH2-containing effectors, forming interactions with motility-related proteins such as Crk. Following the substrate domain is the serine-rich region (SRR), which forms a four-helix bundle. This acts as a protein-interaction motif, and is the site where the serine/threonine phosphatase PP2A interacts with p130Cas. The remaining carboxy-terminal sequence contains a bipartite Src-binding domain (residues 681–713) able to bind both the SH2 and SH3 domains of Src. The coordinated coupling and uncoupling of p130Cas–Crk complexes is critical for motility (Bouton et al., 2001, Barrett et al., 2013, Shin et al., 2004). ABL negatively regulates cell migration by uncoupling p130Cas-Crk complexes. ABL phosphorylates tyrosine residue Y221 on Crk which results in decreased p130Cas-Crk association (Kain and Klemke, 2001).

The regulation of p130Cas, via phosphorylation and dephosphorylation, has downstream consequences that govern many cellular processes. In particular, p130Cas has been demonstrated to play a critical role in cell motility (**Fig. 1.2**). p130Cas becomes phosphorylated via RTK stimulation and upon integrin interaction with ECM components, including fibronectin, vitronectin, laminin and collagen. p130Cas exists at focal adhesions in a macromolecular complex with FAK and Src. Src directly phosphorylates p130Cas in response to numerous growth factors, as well as in response to integrin-mediated stimulation. The assembly results in the activation small GTPases, bringing about changes in the actin cytoskeleton to facilitate cell motility. Phosphorylation of p130Cas and p130Cas-Crk coupling leads to activation of Rac1, which promotes membrane ruffling, lamellipodium formation and actin reorganisation (Barrett et al., 2013, Sharma and Mayer, 2008). In glioma cells, tyrosine residue Y410 is phosphorylated in response to PDGF and HGF and is important in driving glioma cell motility (**Fig 1.2.1.2.2**) (Evans et al., 2011).

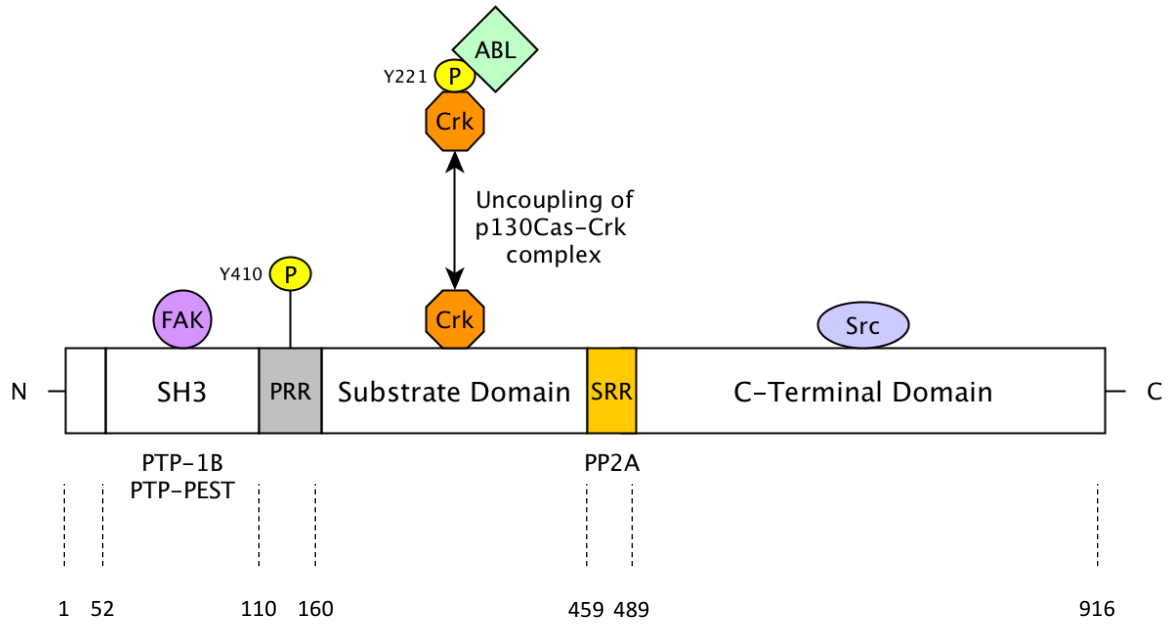


Figure 1.2.1.2.2 Structure of p130Cas. The adaptor protein p130Cas lacks a kinase domain but is made up of several domains that serve as docking sites for various proteins. FAK binds p130Cas via its amino-terminal SH3 domain. Src binds p130Cas via its carboxy-terminal binding domain. Src is responsible for the direct phosphorylation of YxxP motifs in the p130Cas substrate domain, which enables p130Cas to interact with Crk. ABL regulates p130Cas– Crk coupling, by phosphorylating (P) the inhibitory tyrosine Y221 of Crk which results in complex uncoupling. p130Cas is regulated by the phosphatases protein tyrosine phosphatase-1B (PTP-1B) and PTP-PEST that interact with the p130Cas SH3 domain. The serine / threonine phosphatase PP2A regulates serine dephosphorylation via interaction with the p130Cas serine-rich region (SRR). Human p130Cas isoform 1 contains 916 amino acids and the amino acid sequence numbers are represented beneath the figure (Barrett et al., 2013). Phosphorylation (P) at tyrosine residue Y410, located in the proline-rich region (PRR) is important for driving glioma cell motility (Evans et al., 2011). *[Figure adapted from (Barrett et al., 2013)]*

The importance of p130Cas in mediating cell migration is exemplified by the severe consequences caused by aberrant protein expression or activation. p130Cas$^{-/-}$ embryos die *in utero* and show systemic congestion and growth retardation. p130Cas$^{-/-}$ primary fibroblasts contain disorganised, short actin filaments (Honda et al., 1998), and show decreased migration in wound healing assays and decreased invasion through a three-dimensional collagen matrix (Bouton et al., 2001, Honda et al., 1999). p130Cas$^{-/-}$ mouse embryo fibroblasts also exhibit cell spreading and migration defects that are rescued by wildtype p130Cas but not by p130Cas substrate domain-deletion mutants (Shin et al., 2004). Increased expression of p130Cas has been found to correlate with more advanced stages of disease in several cancers, including breast, prostate and ovarian. p130Cas functions as a crucial mediator of transformation in several cancers, including breast cancer and chronic myeloid leukaemia (CML) (Cabodi et al., 2010, Dai et al., 2011, Barrett et al., 2013). Our lab has previously shown that tyrosine phosphorylation of p130Cas plays a critical role in PDGF-induced migration of U87MG glioma cells and vascular smooth muscle cells (Barrett et al., 2014); and that p130Cas activity is important for three-dimensional glioma cell invasion (Frankel et al., 2008). Increased tyrosine phosphorylation of p130Cas has also been detected in glioma cells in response to VEGF and HGF (Evans et al., 2011). Tyrosine residue Y410 of p130Cas is phosphorylated in response to PDGF- and HGF-mediated migration of U87MG glioma cells (Evans et al., 2011).

1.2.1.3 The ERK signalling pathway

The RAS–ERK (mitogen-activated protein kinase 1, also known as MAPK) signalling pathway is made up of the RAS small guanine-nucleotide binding protein and the protein kinases RAF, MEK, and ERK (**Fig 1.2**). RAS is attached to the plasma membrane and can be activated by growth factor, cytokine, and hormone receptors. Activated RAS recruits RAF to the membrane, which subsequently phosphorylates and activates MEK, which then phosphorylates and activates ERK (Heidorn et al., 2010, Viala and Pouyssegur, 2004).

There is growing evidence that the ERK MAPK signalling is implicated in cell motility. ERK has been found to be a target of Src-FAK signalling and found to be associated with PXN near the membrane. ERK contributes to cell motility by regulating small GTPases Rac1 and RhoA, which regulate actin cytoskeletal and adhesion dynamics, and enable the formation of cellular protrusions (Viala and Pouyssegur, 2004).

1.2.2 Tyrosine kinases and phosphatases

Protein phosphorylation regulates virtually every aspect of eukaryotic cell biology. It is a reversible and dynamic process, and is mediated by protein kinases and phosphoprotein phosphatases. Protein tyrosine phosphorylation is fundamental to the maintenance of many biological functions, including gene expression, cell growth, differentiation, migration, adhesion, and apoptosis (Soulsby and Bennett, 2009).

Protein kinase enzymes are classified into the following categories based on the residues which they phosphorylate: protein-serine/threonine kinases, protein-tyrosine kinases, or dual specificity kinases (e.g. MEK) (Roskoski, 2005). Tyrosine kinases (TKs) are a family of enzymes that selectively catalyse the phosphorylation of tyrosine residues in different substrates using ATP. The human kinome contains 518 kinases, of which 90 are protein tyrosine kinases (Jabbour and Lipton, 2013, Manning et al., 2002, Hantschel et al., 2008).

Intracellular signalling pathways mediated by tyrosine phosphorylation are controlled through the balanced and opposing actions of TKs and protein tyrosine phosphatases (PTPs). Whereas kinases transfer a phosphate group from ATP to the hydroxyl group of serine, threonine and tyrosine residues, PTPs hydrolyse the phosphoester bond resulting in protein dephosphorylation (Lillo et al., 2014). It is critical to maintain homeostatic control of tyrosine phosphorylation and the imbalance between TK and PTP activity results in the development of diverse pathophysiological conditions, including cancer and metabolic, neural and immunological disease (Soulsby and Bennett, 2009, Lee et al., 2014b).

Kinases are defined by their ability to catalyse the transfer of the terminal phosphate of ATP to substrates, most often on serine, threonine or tyrosine residues. Kinases typically share a conserved arrangement of secondary structures, arranged into 12 subdomains that fold into a bi-lobed catalytic core, with ATP binding in a deep cleft located between the lobes. All kinases have a conserved activation loop which can assume a number of conformations, from catalytically competent and usually phosphorylated ("active conformation"), through to the "inactive conformation" in which the loop blocks the ATP binding site (Zhang et al., 2009).

1.2.2.1 Receptor tyrosine kinases

Tyrosine kinases are primarily classified into receptor tyrosine kinases (RTKs) e.g. EGFR, VEGFR, PDGFR, and non-receptor tyrosine kinases (NRTKs) e.g. Src, ABL, FAK (Paul and Mukhopadhyay, 2004). 90 tyrosine kinases have been identified in the human genome, 58 of them encoding RTKs and the remainder encoding NRTKs. Tyrosine kinases play an important role in the development of disease, representing a common class of oncogenes and found deregulated in most types of cancerous malignancies (Robinson et al., 2000).

RTKs are cell surface transmembrane receptors that display enzymatic activity. All RTKs have an extracellular ligand binding domain, which is connected to the cytoplasmic domain by a transmembrane helix. With the exception of the insulin receptor, all known RTKs exist as monomers in the cell membrane. They are activated by ligand binding to the extracellular domain which enables dimerisation and trans-phosphorylation in the cytoplasmic domain. The autophosphorylation is critical for the recruitment and activation of effector signalling proteins. Most of the phosphorylated tyrosine residues are located in the non-catalytic regions of the receptor, and function as binding sites for SH2 (Src homology 2) and PTB (phosphotyrosine binding) domains of a variety of proteins. The recruitment of proteins to these sites enables the formation of signalling complexes that enable downstream signalling. RTKs are critical in mediating most fundamental cellular functions, including the cell cycle, migration, metabolism, survival, differentiation and proliferation (Schlessinger, 2000).

1.2.2.2 Non-receptor tyrosine kinases

NRTKs are cytoplasmic proteins, whose activation is more complex, involving heterologous protein-protein interaction (Paul and Mukhopadhyay, 2004). NRTKs regulate many physiological processes such as cell migration, differentiation, proliferation, and survival, by interacting with and phosphorylating a large number of substrates simultaneously. Three NRTKs of interest to this study implicated in tumour progression are discussed below.

1.2.2.2.1 Src

The Src family of protein tyrosine kinases (SFKs) is made up of nine proteins, of which Src is the founding member. SFKs play a key role in mediating signal transduction by a variety of cell surface receptors, including growth factor receptors and integrins. SFKs regulate many fundamental cellular processes, including cell survival, growth, differentiation, and motility (Parsons and Parsons, 2004). In 1911 Rous provided evidence that avian sarcoma could be transmitted between chickens by a non-cellular element (Rous, 1911), subsequently identified as the retrovirus now known as the Rous sarcoma virus. The first recognised transforming protein, v-Src, was identified as the gene encoded by Rous sarcoma virus. This led to the discovery of its cellular proto-oncogene homolog in the vertebrate genome, c-Src, and the discovery that both v-Src and c-Src are tyrosine kinases (Martin, 2001, Jones et al., 2000).

Subsequent research revealed that Src activity is regulated by tyrosine phosphorylation; and that the SH2 and SH3 domains of Src mediate protein–protein interactions with sequences containing phosphotyrosine and proline-rich motifs. Research also revealed that Src was a member of a large family of nine structurally related kinases: the ubiquitously expressed Fyn, Yes and Yrk; Lck expressed in the brain and T cells; Lyn expressed in the brain, B cells and myeloid cells; Blk, Fgr, Hck, expressed in B cells and myeloid cells. SFKs share a conserved domain organisation and are made up of a N-terminal segment, followed by SH3, SH2, linker and tyrosine kinase domains, and a short C-terminal tail (Parsons and Parsons, 2004).

Src kinase activity is regulated by phosphorylation of two tyrosine residues, the activating Y416 residue and the inhibitory Y527 residue. Autophosphorylation at Y416 in the

activation loop promotes kinase activity, whereas phosphorylation of Y527 by the cytoplasmic tyrosine kinase C-terminal Src kinase (CSK) inactivates Src. When Y527 is phosphorylated by CSK, Src undergoes a conformational change whereby Y527 interacts with its own SH2 domain rendering the protein catalytically inactive (Jones et al., 2000, Roskoski, 2005).

Src is located in the cytoplasm at sites of integrin clustering. Following integrin receptor activation, FAK is autophosphorylated and recruits Src via its SH2 domain, and recruits p130Cas via its SH3 domain. This enables the formation of the Src-FAK-p130Cas complex. Src is a critical player of the cell motility pathway that activates several downstream signalling proteins. Amongst the substrates of Src is Src itself (autophosphorylation at tyrosine Y416), FAK, p130Cas and PXN (Sharma and Mayer, 2008, Jones et al., 2000).

Src mediates cell-cell adhesion by interacting with p120-catenin, which subsequently interacts with cadherins (Parsons and Parsons, 2004). Src regulates the activation of Rho-family GTPases and increases the synthesis and secretion of proteases that degrade the extracellular matrix (Martin, 2001). Src also phosphorylates PI3 kinase (PI3K), which is involved in growth factor receptor-mediated signalling (Brown and Cooper, 1996). Src phosphorylates ABL to activate Rho GTPase, which stimulates actin-myosin contractility. In this way Src can promote transforming signalling cascades that result in augmented cell migration. Src activates ABL by phosphorylating its tyrosine residue Y412 in the activation loop of the catalytic SH1 domain (Krishnan et al., 2012).

Src has a role in a variety of signal transduction pathways which result in many endpoints important for the development of malignancy including cell motility, cell invasion, cell adhesion, and cell spreading (Jones et al., 2000). When deregulated, Src activity can influence the transformation of healthy cells into malignant ones. Src enhances the mitogenic response of the EGFR to EGF, and upregulated Src activity is an early event in colon carcinogenesis (Martin, 2001). Importantly, FAK has been found to cooperate with Src family kinases to regulate invasion (Frame, 2002). FAK was initially discovered in the early 1990s as a tyrosine-phosphorylated protein in Src-transformed cells. FAK activates signalling pathways associated with cell adhesion, mitogenesis, and oncogenic transformation (Jones et al., 2000). p130Cas, the binding partner of FAK, was also first identified as a tyrosine-phosphorylated protein in Src-transformed cells (Frame, 2002).

1.2.2.2.2 FAK

Much progress has been made in the last two decades since the initial identification of focal adhesion kinase (FAK) as a highly phosphorylated substrate of the viral Src oncogene product v-Src localised to the integrin cluster of focal adhesions (Hanks et al., 1992, Schaller et al., 1992).

FAK is ubiquitously expressed and functions as a cytoplasmic non-receptor tyrosine kinase as well as a scaffold protein, mediating signals initiated at sites of integrin-ECM attachment and signals triggered by growth factor receptors. The protein is now recognised to play a critical role in diverse signalling pathways that regulate focal adhesions, cell adhesion, cell migration, cell survival and transformation. Its role across these numerous signal transduction pathways can result in enhanced tumour progression, and FAK expression is increased in many cancers, such as breast and prostate (Jones et al., 2000, Slack-Davis et al., 2007, Lee et al., 2014a).

FAK is a 125-kDa protein composed of an N-terminal FERM domain, a central kinase domain, proline-rich regions and a C-terminal focal-adhesion targeting (FAT) domain. The FERM domain mediates the recruitment of FAK to membrane-linked proteins, including RTKs such as EGFR and PDGFR. FAK contains three proline-rich regions (PRR1–3) that bind SH3 domain-containing proteins. The C-terminal PPR2 and PRR3 bind p130Cas (**Fig. 1.2.2.2.1**)(Mitra et al., 2005).

Early studies found that FAK could be activated either by the ECM or by growth factors. FAK activation leads to autophosphorylation at its tyrosine residue Y397 (Nakamura et al., 2001). FAK influences both focal adhesion assembly and disassembly. Phosphorylation of FAK in response to integrin engagement leads to the formation of phosphotyrosine docking sites for a number of signalling molecules involved in motility. The phosphorylation on tyrosine Y397 creates a high-affinity binding site for the SH2 domain of Src family kinases and leads to the recruitment and activation of Src (Parsons, 2003, Jones et al., 2000). Subsequent Src phosphorylation of FAK at Y576 and Y577 promotes maximal FAK activity. Active FAK–Src enables binding of p130Cas SH3 domain to FAK and subsequent phosphorylation of p130Cas by FAK (Tachibana et al., 1997, Mitra et al., 2005). Src phosphorylation of the Y861 site enhances FAK binding to p130Cas (Lee et al., 2014a,

Mitra et al., 2005). As well as directing the phosphorylation of p130Cas, FAK also directs the phosphorylation of PXN. Tyrosine phosphorylation of FAK at residue Y861 is strongly observed during cell migration, and Y861-phosphorylated FAK localises with phosphorylated forms of PXN and p130Cas in mouse mammary gland cells (Nakamura et al., 2001). In transformed mammary epithelial cells, FAK deletion leads to impaired migration, invasion and spreading. This is accompanied by reduced phosphorylation of Src, PXN and p130Cas (Lahlou et al., 2012).

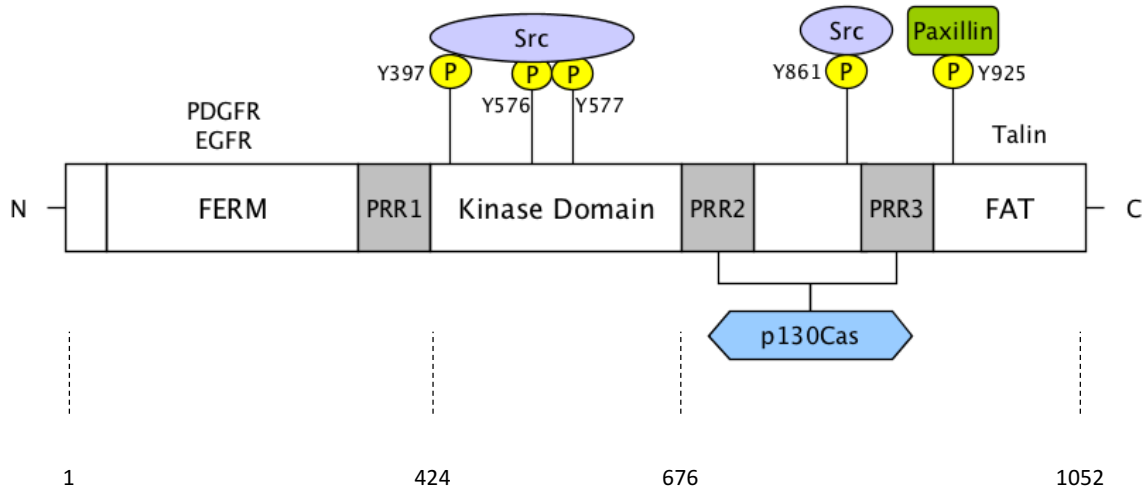


Figure 1.2.2.2.1 Structure of focal adhesion kinase. FAK contains an N-terminal FERM domain, a central kinase domain and a C-terminal focal adhesion targeting (FAT) domain. Human FAK isoform 1 contains 1052 amino acids and the amino acid sequence numbers are represented beneath the figure (Golubovskaya and Cance, 2007). The FERM domain mediates interactions of FAK with receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR). FAK is autophosphorylated (P) on Y397 in response to integrin or growth factor stimulation. This creates a docking site for the SH2 domain of Src. Src-mediated phosphorylation of FAK at Y576 and Y577, which promotes FAK catalytic activity. Src phosphorylation of the Y861 site enhances FAK binding to p130Cas. Active FAK-Src enables p130Cas binding to FAK via the p130Cas SH3 domain. Three proline-rich regions (PRR1–3) bind SH3 domain-containing proteins. PRR2 and PRR3 bind p130Cas. Src phosphorylates FAK at Y861, and this is associated with increased binding of p130Cas to the PRR2 and PRR3 regions of FAK. (Mitra et al., 2005, Nakamura et al., 2001). *[Figure adapted from (Mitra et al., 2005)]*

1.2.2.2.3 ABL

Abelson tyrosine kinase (ABL, also known as c-Abl) is a NRTK that has a role in the regulation of the actin cytoskeleton important for various cellular functions including cell adhesion, proliferation, growth, and development (Kain and Klemke, 2001). Importantly, ABL negatively regulates motility by inhibiting p130Cas–Crk coupling. p130Cas-Crk complexes form in response to integrin or growth factor receptor stimulation via FAK and/or Src activation. The formation of the complex occurs through the interaction of the SH2 domain of Crk with the phosphotyrosine residues in the substrate domain of p130Cas. ABL interacts with the SH3 domain of Crk and phosphorylates Crk inhibitory tyrosine Y221 (**Fig. 1.2.1.2.2**). This causes Crk to functionally fold such that the SH2 domain binds its phosphorylated Y221 residue, prohibiting further p130Cas interaction (Kain and Klemke, 2001). This is reversed by phosphatase PTP-1B- mediated Crk dephosphorylation (Barrett et al., 2013).

However, the role of ABL in cell migration across different cell types remains unclear. Embryonic fibroblasts isolated from animals genetically deficient for ABL showed significantly enhanced cell migration and increased p130Cas-Crk complexes (Kain and Klemke, 2001). In smooth muscle cells, ABL has been reported to mediate cell motility via interaction with β_1-integrin. This leads to actin filament rearrangement and stimulates cell migration. Conversely, actin cytoskeleton remodelling promotes recruitment of ABL to the cell edge (Cleary et al., 2014). There is also evidence that ABL promotes motility of myeloid cells (Baruzzi et al., 2010) and cancer cells (Khusial et al., 2010).

The constitutively active BCR-ABL fusion protein is the cause of disease in ~95% of patients with CML (Seeliger et al., 2007). CML is a stem cell disease caused by the translocation between band q34 of chromosome 9, which contains the ABL proto-oncogene, and band q11 of chromosome 22, which contains the breakpoint cluster region (BCR) gene (Jabbour and Lipton, 2013). The expression of the Philadelphia chromosome [(t9;22)(q34;q11)] and fused oncoprotein BCR-ABL results in constitutive ABL tyrosine kinase activity, critical for the oncogenic transformation of haematopoietic stem cells (Hantschel et al., 2008).

1.2.2.3 Protein phosphatases

Signalling mediated by tyrosine phosphorylation is tightly regulated by the opposing actions of TKs and PTPs. PTPs hydrolyse the phosphoester bond to dephosphorylate proteins (Lillo et al., 2014). Protein phosphatases have been less studied than protein kinases and were originally considered housekeeping enzymes with no important regulatory functions. However, PTPs are now recognised as important regulators, enabling the homeostatic control of phosphorylation and dephosphorylation.

p130Cas is regulated by the protein tyrosine phosphatase-PEST (PTP-PEST; also known as PTPN12). PTP-PEST associates with the SH3 domain of p130Cas and dephosphorylates phosphotyrosine residues, resulting in decreased p130Cas–Crk association and decreased pro-migratory signalling. Cells expressing activated PTP-PEST exhibit defects in cell migration that coincide with significantly reduced levels of tyrosine phosphorylated p130Cas (Barrett et al., 2013, Defilippi et al., 2006). PTP-PEST also regulates cell spreading and migration via interaction with PXN. PTP-PEST binds PXN in fibroblasts and signals through PXN tyrosine Y31 and Y118 residues. PXN is essential for PTP-PEST-dependent regulation of cell spreading and motility in these cells (Jamieson et al., 2005).

PTP1B (also known as PTPN1) plays a role in integrin-mediated adhesion signalling, and its absence in fibroblasts results in attenuated cell spreading (Cheng et al., 2001). PTP1B is a cytosolic phosphatase that is expressed in most cells and functions as a Src Y527 phosphatase (Roskoski, 2005). PTP-PEZ (also known as PTPN14) is also known to interact with p130Cas (Barrett et al., 2013). The SHP-2 tyrosine phosphatase (also known as PTPN11 mediates activation of Ras and MAPK by various mitogens and cell adhesion. Inhibition of SHP-2 has been found to increase the rate of cell spreading, which was accompanied by the enhancement of adhesion-induced tyrosine phosphorylation of PXN and p130Cas (Inagaki et al., 2000). PTEN phosphatase expression leads to tyrosine dephosphorylation of FAK and inhibition of cell migration. Overexpression of FAK partially antagonises the effects of PTEN (Bouton et al., 2001, Tamura et al., 1998).

1.3 Tyrosine kinases in cancer

Approximately 518 kinases are encoded in the human genome and they are implicated in nearly all signal transduction pathways, suggesting that targeting kinase activity therapeutically could elicit a physiological response (Zhang et al., 2009). Research has generated a substantial amount of evidence to show that receptor tyrosine kinases (RTKs) are involved in the processes that govern the transformation of normal cells to malignant cells. When mutated, overexpressed, or structurally altered, RTKs can become 'oncogenic' and stimulate downstream signalling responsible for enhanced migration, proliferation, angiogenesis and survival of cancer cells (Dong et al., 2011, Paul and Mukhopadhyay, 2004).

Aberrant RTK activation in transformed cells has been discovered in a large number of human cancers, including breast cancer, gastrointestinal stromal tumours, non-small-cell lung cancer (Gschwind et al., 2004). Notable examples include the human epidermal growth factor erbB-2 tyrosine kinase (HER2) that is amplified in 20% of breast cancer (Osborne et al., 2004), and EGFR that is implicated in breast, lung and glioma malignancies (Zhang et al., 2009). EGFR, PDGFRα, PDGFRβ, c-Kit and c-Met, have all been reported to be altered in GBM, with EGFR altered at the highest frequency (>40%) (Jones et al., 2000, Lee et al., 2014a, Roth and Weller, 2014, Farin et al., 2006). Because RTK activity is often implicated in the pathology of disease, particularly in tumour progression, targeting them can have significant therapeutic benefit in the treatment of malignancy.

1.3.1 Targeting cancer with small-molecule kinase inhibitors

Considerable efforts have been made to develop small-molecule inhibitors targeting kinases for a number of disorders, most notably for cancer. Between 2001, when the first small-molecule kinase inhibitor imatinib was developed, and 2015, 28 small-molecule kinase inhibitors were approved by the U.S. Food and Drug Administration (FDA), the majority of which are tyrosine kinase inhibitors (TKIs) (Wu et al., 2016). There are over 80 inhibitors that have been advanced to some stage of clinical evaluation (Zhang et al., 2009). Most of the kinase inhibitors developed compete with ATP for the ATP-binding site. The majority of these ATP-competitive inhibitors are Type 1 inhibitors, meaning that they recognise the active conformation of the kinase. The other type of competitive inhibitors are Type 2 inhibitors, which recognise the inactive conformation of the kinase. Movement of the activation loop to the inactive conformation exposes an additional hydrophobic binding site directly adjacent to the ATP-binding site. Conformational shapes are numerous, and the active site can be modelled to accommodate a variety of inhibitors (Zhang et al., 2009).

However, because of the high level of sequence conservation of the ATP-binding site and the large number of kinases, it has proven difficult to design kinase-selective Type 1 and Type 2 competitive inhibitors. There are exceptions, such as lapatinib which targets HER2; but on the whole, inhibitors have a spectrum of targets that increases as their concentration increases (Liu et al., 2013). The third class of kinase inhibitors are allosteric inhibitors that bind outside the ATP-binding site. These inhibitors are typically more selective because they make use of binding sites that are unique to the kinase (rather than the conserved activation loop). The fourth class of inhibitors are covalent inhibitors that irreversibly block ATP binding to the kinase. Although irreversible kinase inhibitors have been designed, there is concern over their use in the clinic due to the possibility of toxicity that can arise if unexpected targets are also irreversibly blocked (Zhang et al., 2009).

1.3.2 Chronic myeloid leukaemia

Chronic myeloid leukaemia (CML) is a myeloproliferative disease characterised by the proliferation of myeloid cells in the bone marrow and blood. CML accounts for about 20% of adult leukaemias, with about 5000 cases each year in the United States (Packer et al., 2011, O'Dwyer and Druker, 2001). CML is a stem cell disease in which neoplastic cells express a specific chromosomal translocation (9; 22) (q34; q11). This translocation between the long arms of chromosomes 9 and 22 generates a shortened chromosome 22, referred to as the Philadelphia (Ph) chromosome (O'Dwyer and Druker, 2001). The molecular consequence from this translocation is the chimeric BCR-ABL gene consisting of the ABL gene fused head-to-tail with 5' exons of the BCR gene (Heisterkamp et al., 1990).

The Ph chromosome results in the expression of the fused oncoprotein BCR-ABL. BCR-ABL is expressed in all patients with Ph chromosome-positive (Ph+) CML, and its expression in animal models identified it as the sole oncogenic event that induces leukaemia. The infection of murine bone marrow with a retrovirus encoding BCR-ABL and subsequent transplantation into irradiated mice was sufficient to induce a myeloproliferative syndrome that resembles the chronic phase of human CML (Daley et al., 1990). Similarly, transgenic mice expressing BCR-ABL develop and die of leukaemia (Heisterkamp et al., 1990).

The constitutive tyrosine kinase activity of BCR-ABL is essential for the transforming properties of the protein. The fusion of ABL and BCR drives transformation and leukaemogenesis via upregulated phosphorylation of substrates such as Crk and activation of pathways such as the RAS/RAF/MEK/ERK and PI3K/Akt pathways. This results in cell proliferation, prevention of apoptosis and subsequent CML cell survival (Packer et al., 2011, Puissant et al., 2012, Steelman et al., 2004).

CML follows a predictable progression involving three distinct clinical stages: chronic phase, accelerated phase, and blast crisis. The chronic phase lasts 3-4 years and is characterised by myeloid hyperplasia, with excessive production of differentiated granulocytes. This is followed by acceleration of the disease (6-28 months) that ultimately leads to the terminal acute blast phase. The blast crisis stage lasts several months, during which patients accumulate excessive amounts of immature cells of the myeloid lineage known as blast cells (Dick, 2008, Calabretta and Perrotti, 2004).

1.3.3 Target-based drug discovery for the treatment of chronic myeloid leukaemia

The identification of the leukaemic oncogene BCR-ABL as the causative molecular pathogenetic event in Ph+ CML provided the rationale for targeting the protein for the treatment of the disease and allowed for the development of the first fusion gene-targeted drug, imatinib mesylate (Gleevec®, STI-571). The rationally designed tyrosine kinase inhibitor, imatinib, was approved in 2002 for the treatment of CML in any phase, and remains frontline therapy. Imatinib blocks the ATP-binding site of BCR-ABL, thereby preventing ATP from binding. This inhibits BCR-ABL tyrosine kinase activity and blocks downstream signalling (Druker, 2008).

Prior to the approval of imatinib, the standard therapy for CML consisted of recombinant interferon-α alone, or in combination with the chemotherapy agent cytarabine. However, this combined approach led to a major cytogenetic response in only 6-25% of patients (Waller, 2010). The cytogenetic response (CyR) refers to the reduction of cells in the bone marrow expressing the fused Ph chromosome (O'Brien et al., 2003).

A phase I clinical trial with imatinib was launched in 1998 to determine the maximally tolerated dose and to delineate side effects, with clinical benefit as the secondary endpoint. Patients with chronic phase CML who had failed therapy with interferon-α were enrolled (Druker et al., 2001). Three open-label phase II studies were subsequently initiated using imatinib as a single therapeutic and looking at over one thousand Ph+ CML patients in chronic phase, accelerated phase, and blast crisis. In 532 patients with chronic phase CML who had failed interferon-α therapy, 95% of patients reached a complete haematologic response, that is the normalisation of white blood cell counts. Over 80% of patients in the accelerated phase showed some haematologic response, with over 50% reaching complete haematologic response. Over 50% of patients in blast crisis exhibited some haematologic response to imatinib. The results of these phase I and phase II trials led to the U.S. FDA approval of imatinib in 2001 for the treatment of CML in advanced phase and after failure of interferon-α therapy (Waller, 2010, O'Brien et al., 2003).

The landmark IRIS (International Randomized Study of IFN and STI571) open-label phase III clinical trial looked at the combination of interferon-α and cytarabine compared with

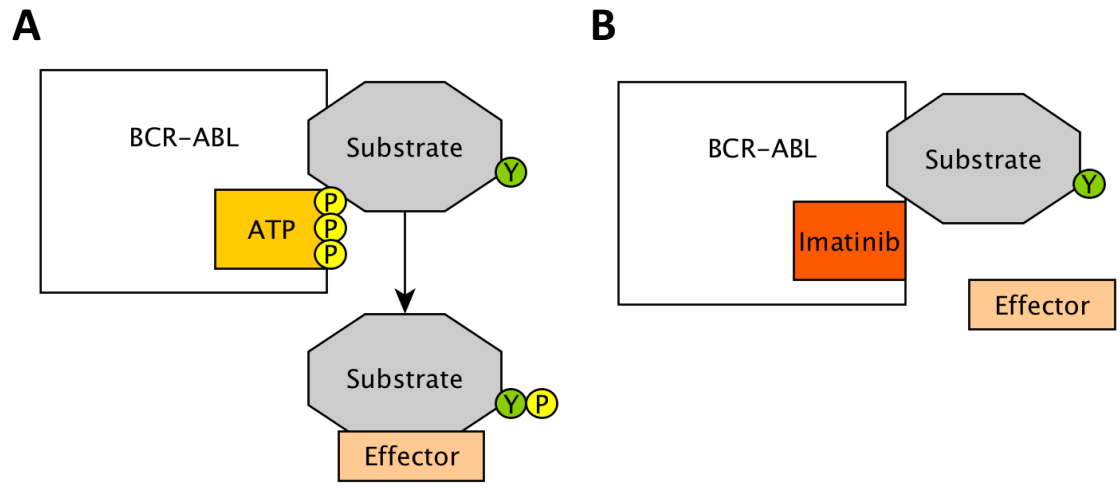
imatinib. 1106 patients across 16 countries were randomly assigned to treatment with either 400 mg/day of imatinib, or interferon-α and cytarabine (553 in each group). After a median follow-up of 19 months, patients treated with imatinib had significantly better complete haematologic response, major cytogenetic response, and complete cytogenetic response (CCyR), meaning that no cells with the Ph+ chromosome were found in the bone marrow. This was accompanied by a significantly better progression-free survival compared to patients treated with the traditional anticancer agents (O'Brien et al., 2003). Study results were disclosed early as a consequence of such promising outcomes and most patients being treated with interferon-α and cytarabine were transferred over to the imatinib group (Waller, 2010).

In the IRIS five-year follow-up study, the 553 patients in the initial imatinib group were assessed for response to the tyrosine kinase inhibitor. It was found that by 60 months, 87% of patients achieved CCyR. Importantly, nearly all patients (97%) with a CCyR within 12 months after starting treatment did not progress to the accelerated phase or blast crisis by 60 months. The estimated overall survival rate at 60 months was 89%. As well as a significantly positive response to imatinib, patients exhibited acceptable side effects with few severe grade 3 or 4 adverse effects (Druker et al., 2006).

It took less than 15 years from the identification of the critical role of the BCR-ABL oncoprotein to develop imatinib, which is now the standard of care for patients with CML. Imatinib provided validation that targeting kinases with the newer class of anticancer drugs, the so-called "small-molecule" inhibitors, could have significant therapeutic benefit without major side effects. The gene-to-drug approach employed in the development of imatinib has validated the impact of rational target-based drug design for the treatment of cancer and has raised expectations for subsequent drug discovery.

In the absence of imatinib, ATP is bound to BCR-ABL and transfers its terminal phosphate to a substrate, which becomes activated and able to bind downstream effectors (**Fig. 1.3.3A**). Imatinib is a Type 2 inhibitor, binding with high affinity to the ATP binding site in the inactive form of the tyrosine kinase domain. Imatinib prevents ATP from binding, and leads to the inhibition of kinase activity by preventing the transfer of phosphate to tyrosine residues on substrate proteins (**Fig. 1.3.3B**) (Waller, 2010). *In vitro* studies have shown that imatinib potently inhibits all of the ABL tyrosine kinases, including ABL, viral ABL (v-

ABL), BCR-ABL, as well as the closely related kinase ARG (also known as ABL2). Imatinib also potently inhibits the stem cell growth factor receptor (c-Kit), platelet derived growth factor receptor beta (PDGFRβ), the collagen receptor discoidin domain receptor tyrosine kinase 1 (DDR1), and the non-kinase NAD(P)H:quinone oxidoreductase NQO2 (**Fig. 1.3.3C**) (Rix et al., 2007, Hantschel et al., 2008, Deininger et al., 2005).



C

TARGETS OF IMATINIB

ABL
ARG
BCR-ABL
KIT
PDGFR
DDR1
NQO2

Figure 1.3.3 Mechanism of action of imatinib. (**A**) ATP occupies the pocket of the ABL component of BCR-ABL and is able to transfer its terminal phosphate (P) to a substrate, phosphorylating an activating tyrosine (Y) residue. The substrate is activated, detaches from BCR-ABL and makes functional contact with a downstream effector. (**B**) When imatinib occupies the ATP binding site, it prevents the phosphorylation and subsequent activation of the substrate. The substrate cannot bind downstream effectors and downstream signalling pathways that would otherwise promote cell proliferation and cell survival are inhibited. (**C**) The known targets that imatinib binds to with high specificity.

1.3.4 Development of resistance to imatinib in chronic myeloid leukaemia

The development of a highly selective and safe drug shifted the paradigm for molecular targeted cancer therapy – it showed that you can successfully target an oncoprotein that is the driver of malignancy to effectively treat disease without severe side effects. Imatinib is the current standard of care for patients with Ph+ CML. However, a number of patients develop resistance leading to relapse, or intolerance leading to discontinuation of treatment. Despite exhibiting an overall response rate of about 80%, imatinib faces significant challenges in the clinic that are imposed by drug resistance and lack of inhibitor efficacy (Zhang et al., 2009).

The response to imatinib in CML is maintained by selective inhibition of the BCR-ABL ATP binding domain. The most prevalent cause of resistance is the occurrence of point mutations within the kinase domain which interferes with drug binding. Nearly 100 different mutations conferring varying degrees of resistance have been identified *in vitro* and *in vivo* (Quintas-Cardama et al., 2010, Hantschel et al., 2008).

The efficacy of imatinib is astounding for those patients in chronic phase CML, where the rate of complete haematologic response approaches 100%. But for those patients in accelerated phase and blast crises phase, the efficacy of imatinib is significantly lower (Quintas-Cardama et al., 2010). And even though initial response rates to imatinib are high, the TKI fails in up to 40% of patients with prolonged treatment, most often because of mutations in the BCR-ABL kinase domain. Less frequently, imatinib is discontinued as a result of adverse side effects (Cortes et al., 2013). The overall failure rate of imatinib at the 5-year follow up in the IRIS study was 17% (Druker et al., 2006). In the accelerated and blast crisis phases, the combination of high numbers of proliferating tumour cells coupled with genomic instability leads to secondary genetic alterations that are independent of BCR-ABL. The clones that acquire a genetic alteration conferring resistance to imatinib are selected by the drug (von Bubnoff et al., 2002). Three second generation tyrosine kinase inhibitors were developed with enhanced potency towards wildtype BCR-ABL for the purpose of targeting cells resistant to imatinib: nilotinib and dasatinib both now approved for first-line therapy, and bosutinib approved for CML patients with resistance to other TKIs.

1.3.5 Second- and third-generation small-molecule inhibitors for chronic myeloid leukaemia

Nilotinib (Tasigna, AMN107) is the second-generation TKI rationally designed and approved in 2010 as first-line treatment of CML and for those patients that become resistant or intolerant to imatinib. Similar to imatinib, nilotinib is a Type 2 competitive inhibitor, and was designed on the basis of crystallographic studies that looked at the interaction of imatinib and the ABL kinase domain. Nilotinib has an improved affinity and specificity against BCR-ABL when compared to imatinib: it is 20- to 30-fold more potent against wildtype (unmutated) BCR-ABL *in vitro* with an IC_{50} (50% inhibitory concentration) of 25 nm (Jabbour and Lipton, 2013, Quintas-Cardama et al., 2010). Importantly, nilotinib is also strongly active against most imatinib-resistant BCR-ABL mutants harbouring point mutations (Weisberg et al., 2006). Like imatinib, nilotinib also potently inhibits ARG, PDGFR, c-Kit, and DDR1 and NQO2 (Hantschel et al., 2008).

Successful outcomes from phase I and II studies looking at patients with CML resistant or intolerant to imatinib led to the accelerated approval of nilotinib for CML after imatinib failure. The phase I study looking at 119 patients with imatinib-resistant CML established a safe 400 mg twice-daily dose of nilotinib for phase II trials. The same study found that nilotinib induced a CCyR in 53% of chronic phase patients, 48% of accelerated phase, and 27% of blast crisis phase (Quintas-Cardama et al., 2010).

In the phase III, randomised, open-label, multicentre trial, efficacy and safety of nilotinib (300 or 400 mg twice daily) was compared with that of imatinib (400 mg once daily) in a total of 846 patients with newly diagnosed Ph+ CML in the chronic phase. The primary end point was the rate of major molecular response at 12 months, defined as a BCR-ABL transcript level of ≤ 0.1% in the peripheral blood; and the secondary end point was the rate of CCyR at 12 months. By 12 months, rates of major molecular response were significantly higher in patients receiving 300 mg of nilotinib (44%) or 400 mg of nilotinib (43%) twice daily than in those receiving imatinib (22%). The rates of CCyR were also higher among patients receiving 300 mg nilotinib (80%) and 400 mg nilotinib (78%) compared to those receiving imatinib (65%). Severe grade 3 and 4 adverse effects were uncommon in all patients. Overall, nilotinib was superior to imatinib in both primary and secondary end points. And the transformation to the accelerated phase or blast crisis was significantly

lower in both nilotinib groups than in the imatinib group (Saglio et al., 2010). The recent five-year follow up identified that more 50% of patients in the nilotinib groups achieved a molecular response compared to 31% of patients treated with imatinib. But cardiovascular events, and elevations in blood cholesterol and glucose level, were more frequent in patients receiving nilotinib (Hochhaus et al., 2016). However, overall the long-term results support the use of nilotinib 300 mg twice daily as frontline therapy for patients with chronic phase CML.

Nilotinib was active against 32/33 imatinib-resistant BCR-ABL mutants studied, but had no significant effect against the T315I mutant (Weisberg et al., 2006). The T315I mutation confers resistance against imatinib, and second-generation TKIs nilotinib, dasatinib and bosutinib. This is because the "gatekeeper" T315 residue is located within the ABL kinase binding site and mediates access to the hydrophobic pocket to which the inhibitors bind. The isoleucine residue conferred by the mutation is bulky and obstructs the inhibitors' ability to bind (Quintas-Cardama et al., 2010, Weisberg et al., 2006, Cortes et al., 2012). The so-called "gatekeeper" T315I mutation accounts for 4-20% of all mutations that are associated with TKI resistance, and stem cell transplantation is considered the first-line option for CML patients with the T315I mutation (Xu et al., 2016).

The second-generation TKI dasatinib is approved for patients with Ph+ CML resistant or intolerant to imatinib. Dasatinib is about 300 times more potent than imatinib in cells expressing unmutated BCR-ABL *in vitro*. Dasatinib also potently inhibits SRC-family kinases, c-Kit, PDGFR, DDR1, and ephrin receptor kinase. At least 30 additional kinases have been found to bind to dasatinib and the broad spectrum of targets may account for the unique side effects that have been observed in a large proportion of patients (Lindauer and Hochhaus, 2010, Hantschel et al., 2008). Bosutinib is another dual Src-BCR-ABL kinase inhibitor approved for Ph+ CML resistant or intolerant to imatinib (Keller et al., 2010).

Even second-generation TKIs nilotinib, dasatinib and bosutinib, are not successful in all patients that discontinue imatinib. Between 37-52% of patients treated with the second-generation inhibitors do not respond, and an additional ~25% of patients have an initial major CyR that is lost within 2 years (Cortes et al., 2012, Kantarjian et al., 2011). Second-generation TKIs consistently fail in those patients with the gatekeeper T315I mutation (Jabbour et al., 2015). Ponatinib, a third-generation TKI designed using a computation and

structure-based approach, is active against the T315I mutation. In a phase I trial looking at patients with haematologic cancers for whom no standard care was available, ponatinib caused a complete haematologic response in 98% of patients with chronic phase CML, and a CCyR in 63% of patients (Cortes et al., 2012). In a multi-centre phase II trial, the rate of major CyR in response to ponatinib in patients with the T315I mutation was 70% for chronic phase, 50% for accelerated phase, and 29% for blast crisis phase (Cortes et al., 2013). However, the long-term response and side effects are still not known because of short-term follow up.

1.3.6 Mechanisms underlying resistance

Resistance to TKIs includes primary resistance, i.e. *de novo* resistance, describing patients that do not respond to the drug from the start of treatment and do not achieve a partial remission; and secondary resistance, i.e. relapse after initial response. Both primary and acquired resistance can be caused by alterations to drug metabolism (uptake and efflux) or by modifications of the drug target (Zahreddine and Borden, 2013).

Almost all tumours are heterogeneous, consisting of mixed populations of cells. Acquired resistance can arise due to the presence of pre-existing resistant subclones that survive treatment and expand after therapy, or due to the acquisition of mutations in response to treatment that render certain cells resistant (Oxnard, 2016). Patients with non-small cell lung cancer with activating mutations in the EGFR kinase domain usually respond well to the tyrosine kinase inhibitors gefitinib and erlotinib. However, patients typically develop resistance and relapse after about a year. In a lot of cases resistance is caused by the acquisition of the "gatekeeper" secondary kinase domain mutation, T790M (Godin-Heymann et al., 2008).

Multidrug resistance is a big contributing factor to the development of resistance to anticancer drugs. Anticancer drugs may kill drug-sensitive cells, but the proportion of drug-resistant cells that are left behind will survive, proliferate and eventually render the tumour resistant to treatment (Persidis, 1999). Resistance is correlated to the presence of several drug transporters, the so-called molecular "pumps", that serve as efflux pumps and remove drugs from tumour cells. The pumps commonly found to confer resistance are ATP-binding cassette (ABC) drug transporters: P-glycoprotein (encoded by ABCB1), multidrug resistance-associated protein (encoded by ABCC2) and ABCG2. These are important multidrug-resistance genes, conferring simultaneous resistance to unrelated drugs that do not necessarily have the same mechanism of action (Persidis, 1999, Lou and Dean, 2007).

Cancer stem cells are inherently highly resistant to chemotherapeutic drugs and contribute to resistance. Not only do CSCs possess the ability to self-renew and differentiate into cells that help maintain a tumour, they also express high levels of ABC transporters (Lou and Dean, 2007). For example, ABCG2 is highly expressed in haematopoietic stem cells. It has

been proposed that chemotherapy drugs used in combination with ABC transporter inhibitors would more efficiently target CSCs (Dean et al., 2005).

In imatinib-sensitive Ph+ CML white blood cells, imatinib is available at a sufficient quantity to inhibit all BCR-ABL molecules. Resistance can arise as a result of overexpression of BCR-ABL, whereby some BCR-ABL molecules are not bound to imatinib. In this scenario, the leukaemic cell is able to maintain a baseline level of signalling sufficient for cell survival. Specific mutations within the BCR-ABL kinase domain can also confer resistance by preventing the binding of imatinib and enabling ATP binding. Genomic instability can lead to the acquisition of additional mutations that may contribute to BCR-ABL-independent growth and / or survival of malignant clones (von Bubnoff et al., 2002). Genomic instability, or clonal evolution, leads to gene amplification, mutation, and additional genetic changes in the leukaemic cell, suggesting that genomic instability would contribute to resistance in more than one way.

Another possible reason for the failure of RTK inhibitor monotherapy is the presence of other activated RTKs in the same tumour that are not being targeted by the drug. In this scenario, targeting a single RTK is not effective because other RTKs will drive tumour growth and maintain activation of signalling pathways instead. This phenomenon known as "RTK-switching" supports the idea that combinational therapies may be required for the treatment of malignancy (Inda et al., 2014).

1.3.7 Targeting glioblastoma multiforme with tyrosine kinase inhibitors

The current standard treatment for GBM consists of surgical resection followed by chemotherapy and radiotherapy. However, tumour cells are able to evade surgery and chemoradiotherapy, and recurrence is inevitable, typically occurring within 1-2 cm from the original border (Cuddapah et al., 2014). Following the success of imatinib for the treatment of CML, small-molecule inhibitors that inhibit kinase activity were developed for solid cancers. For example, the EGFR and HER2 inhibitor, lapatinib, was developed for HER2-positive breast cancer, and the RAF, PDGFR, VEGFR and c-Kit inhibitor, sorafenib, for hepatocellular carcinoma (De Witt Hamer, 2010). However, there are still no small-molecule kinase inhibitors for glioblastoma that show significant efficacy.

Tyrosine kinases play a fundamental role in signal transduction that becomes deregulated in GBM. Migration and proliferation of GBM cells can be stimulated by the action of growth factors and their tyrosine kinase receptors, which often appear to be over-expressed or constitutively active in this cancer. The epidermal growth factor receptor (EGFR, also known as HER1 or ERBB1) is amplified, mutated, and/or rearranged in more than 40% of GBM. PDGFRα, PDGFRβ, c-Kit and c-Met, are also reported to be altered in GBM at lower frequencies (Roth and Weller, 2014).

Because GBM remains incurable and kinases play an integral role in driving pathology, the application of small-molecule kinase inhibitors for the treatment of GBM is an obvious strategy. Targeting the EGFR is regarded as a particularly promising therapeutic strategy for GBM and small-molecule inhibitors, such as erlotinib, having been investigated. However, only 10 to 20% of patients benefit from current EGFR-directed strategies (erlotinib, or monoclonal antibodies): mutations, poor tumour perfusion, and the blood-brain barrier all present significant challenges and many patients do not respond or develop resistance (Roth and Weller, 2014).

Overexpression and activation of PDGFR may contribute to the transformed phenotype of gliomas. PDGF A / B ligands and PDGFR α / β receptors are expressed in nearly all cultured glioma cell lines and in astrocytomas resected from patients (Kilic et al., 2000). Dominant-negative PDGF mutants block the proliferation of glioma cells *in vitro* and *in*

vivo (Shamah et al., 1993). In a glioma cell line with constitutively activated PDGF signalling pathway, the application of PDGF antibodies inhibited colony growth, diminished DNA synthesis and reversed the transformed morphology of tumour cells (Vassbotn et al., 1994). However, anti-PDGFR therapy has so-far failed in GBM patients in the clinic (Stommel et al., 2007).

Within the context of inhibiting tyrosine kinase activity, imatinib has been shown to inhibit the growth of glioma cells *in vitro* and *in vivo* in an intracranial GBM mouse model (Kilic et al., 2000). However, despite encouraging clinical results for CML, imatinib has failed clinical trials for glioblastoma, where it shows no significant inhibition of tumour growth or extension of survival. The North American Brain Tumor Consortium phase I/II trial of patients with recurrent malignant glioma found that single-agent imatinib has minimal activity and may be associated with a higher risk of intratumoural haemorrhage (Wen et al., 2006). Another single-arm, phase II trial involving 112 patients with malignant astrocytomas (51 patients with GBM) also found that imatinib shows no significant antitumour activity in patients with recurrent gliomas (Raymond et al., 2008).

As mentioned, only 10 to 20% of patients benefit from current EGFR-directed strategies, and anti-PDGFR therapy has failed in GBM patients (Roth and Weller, 2014, Stommel et al., 2007). The small-molecule imatinib has also failed. In fact, a systemic review of clinical studies looking at the use of small-molecule inhibitors for GBM found that the efficacy of the inhibitors examined does not warrant switching from the standard of care for adult patients (De Witt Hamer, 2010).

GBM is characterised by strong intratumoural heterogeneity, and imatinib is likely to have failed because multiple kinases would be exerting pathogenic effects. Heterogeneous pattern of RTK amplification has been observed in GBM. The three receptors most commonly amplified are EGFR, PDGFR and c-Met, with evidence that tumour cells present varying amplification patterns within a single tumour (Inda et al., 2014). Aberrant kinase receptor activity may condition the response of GBM cells to targeted therapies. The absence of well-known mutations that predict the response to tyrosine kinase inhibitors and the presence of compensatory pathways prevent potent anti-glioma effects of drugs that target these receptors (Roth and Weller, 2014, Stommel et al., 2007).

1.3.8 Imatinib for the treatment of gastrointestinal stromal tumours

Imatinib has also been approved for use in gastrointestinal stromal tumours (GIST), where mutated c-Kit (also known as CD117) has been found to play a critical role in the pathogenesis of the disease. c-Kit is expressed in a variety of human cancers, including neuroblastoma, melanoma, small cell lung cancer, breast and ovarian cancer, as well as GIST (Waller, 2010). For most of these cancers, the precise role of c-Kit is not defined. However, in the case of GIST, gain-of-function mutations in KIT are found in 95% of tumours and these mutations cause the kinase to become a proto-oncogene. c-Kit becomes constitutively active, which leads to uncontrolled cell proliferation and resistance to apoptosis (Demetri et al., 2002, Din and Woll, 2008).

GISTs are soft tissue sarcomas of the gastrointestinal tract, originating from the neoplastic transformation of the intestinal cells of Cajal. KIT mutations result in the constitutive activation of the kinase leading to the activation of downstream effectors that deregulate cell proliferation and survival (Fukuda et al., 2013). The stem-cell factor (SCF) binds the extracellular SCF-binding domain of the transmembrane c-Kit receptor, leading to homodimerization and autophosphorylation of intracellular tyrosine residues. This creates docking sites for signal transduction effectors, leading to the activation of downstream signalling cascades involving MAPK, PI3K and RAS, which mediate c-Kit-induced mitogenesis and differentiation (Joensuu et al., 2002). GISTs range from small non-malignant tumours curable with surgery to aggressive malignant cancers. About 20-30% of GISTs are malignant and the median survival from the time of diagnosis of metastatic or recurrent disease ranges from 12 to 19 months (Dagher et al., 2002, Joensuu et al., 2002).

Surgery is the standard initial treatment for non-metastatic GISTs. For aggressive cancers, the standard of care prior to imatinib was chemotherapy. However, this was ineffective in the treatment of malignant unresectable GISTs. In one study only 7% of GIST patients responded to chemotherapy with two anticancer agents; and in another study involving five anticancer drugs, only 5% of patients responded (Joensuu et al., 2002).

The tumour volume of the first GIST patient ever to receive imatinib shrunk by more than 70% after 8 months of treatment (Joensuu et al., 2001). Imatinib's accelerated approval for

the treatment of advanced or metastatic GIST in February 2002 was based on response rate, an endpoint judged to likely predict clinical benefit. Response rate was calculated by carrying out a 24-month single open-label phase II trial of 147 patients with metastatic and/or unresectable malignant GIST. The study found that 38% of patients responded to imatinib, with pharmacokinetics similar to CML patients (Dagher et al., 2002).

Accelerated approval allows patient access to promising drugs but requires a follow-up study to demonstrate clinical benefit. Imatinib subsequently received accelerated full approval for primary treatment of patients with KIT-positive GIST in 2008 based on the positive findings from two large open-label, multicentre, randomised phase III studies which looked at a total of 1640 patients. The median progression-free survival time was approximately 20 months and the median overall survival time was approximately 49 months (Cohen et al., 2009). The finding that c-Kit expression is altered in GIST and the development of imatinib has revolutionised the diagnosis and treatment of GIST, with overall response rates to the drug in excess of 80% (Din and Woll, 2008).

However, treatment with imatinib is not uniformly successful. Although the 2-year survival rate for imatinib treatment of GIST ranges from 70-80%, between 10 and 20% of patients exhibit primary resistance to imatinib (Bauer et al., 2007, Bauer et al., 2005). The clinical outcome of imatinib is genotype-dependent in regards to both primary and secondary mutations that render c-Kit unresponsive to the TKI. One study that aimed to correlate clinical response to imatinib with tumour genotype found that tumours harbouring mutations in exon 11 were twice as likely to exhibit a partial response to imatinib (≥50% decrease in tumour diameter) than tumours with mutations in exon 9 (Heinrich et al., 2003). A rare mutation in exon 17 has been found to confer resistance to imatinib in one patient and there are other rare mutations whose clinical outcome remain unknown (Spitaleri et al., 2015).

Although more than 80% of patients benefit significantly from imatinib treatment, most of these will eventually develop resistance and their disease will progress. In one study 12 patients were selected for surgery to resect residual disease following imatinib treatment. Out of the 11 specimens from patients for whom complete resection was possible, 10 were found to contain viable tumour cells (Bauer et al., 2005, Bauer et al., 2007). This suggests that imatinib is not able to produce a complete response. Nilotinib is not currently used for the treatment of GIST. The open-label phase III trial of nilotinib versus imatinib as front-

line therapy for GIST patients was discontinued by Novartis in 2011 because interim results indicated that nilotinib would not be superior to imatinib. In a phase III trial of patients with advanced GIST who had previously been treated with imatinib and sunitinib, nilotinib did not show any advantages in progression-free or overall survival compared with renewal of imatinib treatment (Reichardt et al., 2012).

The mechanisms underpinning primary and secondary resistance to imatinib remain unknown. However, most patients with imatinib resistance continue to express activated c-Kit, suggesting that the c-Kit signalling pathway should remain a useful therapeutic target. But alternative small-molecule inhibitors have shown only a minor efficacy when used in GIST patients with secondary imatinib resistance. Secondary KIT mutations acquired during treatment have been reported, particularly in the kinase domain. But it is unclear whether these mutations simply block imatinib binding or whether they also hyper-activate c-Kit, which would lead to hyper-activation of downstream signalling (Bauer et al., 2007).

The limited efficacy of other c-Kit kinase inhibitors may result from heterogeneity of mutations within individual patients. In this scenario any single drug would unlikely inhibit a broad range of mutations found in a patient, suggesting that a combinational approach targeting downstream effectors of c-Kit may help overcome the problem of resistance. The same signalling pathways are activated in imatinib-resistant GISTs as in untreated GISTs, notably the PI3K/AKT and ERK pathways. One approach to tackle imatinib resistance may be to target downstream signalling proteins. This is supported by findings that additive effects have been observed in GIST using imatinib together with either an mTOR inhibitor, PI3K inhibitor, or a MEK inhibitor. In imatinib-sensitive GIST, imatinib-induced inhibition of c-Kit is accompanied by significant decreases in phosphorylation of mTOR, AKT, and ERK (Heinrich et al., 2006). In a subsequent study, mTOR inhibition using a pharmacological inhibitor in conjunction with imatinib produced additive effects, inhibiting proliferation and apoptosis more than when either of the inhibitors were used alone (Bauer et al., 2007). As expected, imatinib induced almost no apoptosis and only minor antiproliferative effects in two GIST cells lines established from patients with imatinib-resistance. However, a PI3K inhibitor induced strong antiproliferative effects (40-75% inhibition of proliferation) and apoptosis (three- to four-fold increase), suggesting that targeting downstream effectors may be of therapeutic benefit for the treatment of imatinib-resistant GIST (Bauer et al., 2007).

1.4 Aims of the project

Many of the motility-related proteins involved in migration are tyrosine kinases, including FAK, Src, c-Abl, and RTKs at the cell membrane. The target spectrum of imatinib and nilotinib include key kinases involved in motility (**Fig. 1.3.3C**). Therefore, the effects of imatinib and nilotinib on GBM motility *in vitro* were investigated. Surprisingly, preliminary results showed increases in tyrosine phosphorylation of the adaptor proteins p130Cas and PXN, and of the tyrosine kinase FAK, in response to stimulation with either imatinib or nilotinib (**Fig 1.4**). The preliminary unforeseen effects of TKIs on glioma cell tyrosine phosphorylation set the foundation of this project. The residues that were identified to be phosphorylated on p130Cas, FAK and PXN play a key role in driving glioma motility. I wanted to identify the effects of imatinib and nilotinib on glioma cellular signalling *in vitro*.

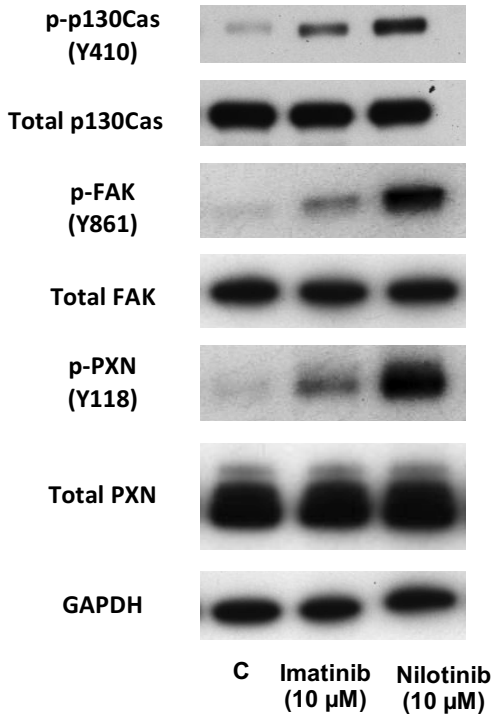


Figure 1.4 Imatinib and nilotinib induce tyrosine phosphorylation of three key motility-related proteins. Cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with either serum free medium (SFM) & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies.

Importantly, despite encouraging clinical results for CML and for GIST, imatinib has failed clinical trials for GBM, where it shows no significant inhibition of tumour growth or extension of survival. The molecular mechanisms of action of imatinib in GBM is still not well understood. Imatinib and nilotinib are reported to cause activation of intracellular kinases including PI3K, Akt and ERK (Fenouille et al., 2010, Mokhtari et al., 2013, Packer et al., 2011, Dong et al., 2011). The activation of alternative pathways may contribute to the lack of clinical efficacy of imatinib in GBM trials. It was proposed that given the preliminary findings of enhanced activation of key motility-related proteins, imatinib and nilotinib may be exerting undesirable stimulatory effects on glioma migration. It was also proposed that using imatinib and nilotinib in a GBM model would help delineate the mechanisms of tumour development driving glioma pathology and would help identify targets for therapeutic intervention.

Although the target selectivity, pharmacokinetic profiles, and toxicity profiles are well defined for imatinib and nilotinib, the precise molecular outcomes of drug treatment are not defined, and subsequently, neither is their clinical management. Inhibition of other TKs and co-activation of signalling pathways may account both for the development of imatinib resistance in Ph+CML and GIST. The aim of this project was to identify the target(s) and alternative downstream pathways being activated by imatinib and nilotinib. Identifying the effects of imatinib and nilotinib on cell functions modulating tumour behaviour is essential for understanding critically important aspects of drug treatment including non-responsiveness, the development of resistance, and the occurrence of side effects. The identification of novel pathways being activated by imatinib and nilotinib could help identify new targets for combinational treatment.

2 Materials and methods

2.1 Materials

2.1.1 General consumables, chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), Ham's F-10 medium, DMEM (10X), Lipofectamine 2000, NuPAGE® Bis-Tris gels, Invitrolon™ polyvinylidene difluoride membranes, NuPAGE® Transfer Buffer, NuPAGE® Electrophoresis Apparatus, Opti-MEM, and 2.5% trypsin solution were from Invitrogen Life Technologies Ltd (Paisley, UK). Bovine serum albumin (BSA), Ethylenediaminetetraacetic acid (EDTA), Foetal bovine serum (FBS), Dimethyl sulphoxide (DMSO), Methanol, NP40, Penicillin-Streptomycin, RIPA, 4X SDS Sample buffer, Phosphatase Inhibitor Cocktail 1, Phosphatase Inhibitor Cocktail 2, TWEEN®20 and Methyl cellulose were from Sigma-Aldrich (St. Louis, USA). Spectra Multicolor Broad Range Protein Ladder was from Thermo Scientific (Waltham, USA). cOmplete Protease EDTA-free Inhibitor Cocktail Tablets were from Roche Applied Science (West Sussex, UK). ECL Western Blotting Detection Reagent and Amersham Hyperfilm ECL were from GE Healthcare (Little Chalfont, UK). Falcon tissue culture dishes, Falcon tissue culture plates, Non-tissue culture treated u-bottom 96 well plates, Transwell inserts, Tissue culture flasks and Bovine Pituitary Extract (354123) were from BD Bioscience (Oxford, UK). MITO+ serum extender (355006) was from Corning Inc. (Corning, USA). PureCol® bovine collagen was from CellSystems Biotechnologie Vertrieb GmbH (Troisdorf, Germany). Paraformaldehyde and Triton X-100 were from Sigma. Vectashield Microscope Mounting Medium with DAPI was from Jackson Immunoresearch Laboratories (Luton, UK).

2.1.2 siRNA

The AllStars Negative Control was purchased from Qiagen (Crawley, UK). Refer to **Table 2.1.2** for a list of all siRNa used.

Table 2.1.2 List of siRNA used.

TARGET GENE	COMPANY	SEQUENCE (5' →3')
ABL1 (#10)	Qiagen	ACGCACGGACAUCACCAUGAA
ABL2 (Arg; #8)	Qiagen	AACCCUGUCCUUAAUAACUUA
BCAR1 (p130Cas; #1)	Life Technologies	GAGUUUGAGAAGACCCGAUU
BCAR1 (p130Cas; #2)	Dharmacon	GGUCGACAGUGGUGUGUAU
BRAF (#1)	Qiagen	AACAUAUAGAGGCCCUAUUGG
CRAF (#5)	Qiagen	AAGACGUUCCUGAAGCUUGCC
CSK	Qiagen	AAGUACAAUUCCACGGCACU
DDR1 (#9)	Qiagen	ACGGUGUGAAUCACACAUCCA
ITGB1 (#1)	Dharmacon	GAACAGAUCUGAUGAAUGA
ITGB1 (#2)	Dharmacon	CAAGAGAGCUGAAGACUAU
ITGB3 (#1)	Dharmacon	CUCUCCUGAUGUAGCACUUAA
ITGB3 (#2)	Dharmacon	CACGUGUGGCCUGUUCUUCUA
PDGFRβ	Qiagen	GGAACGUGCUCAUCUGUGA
PTPN14 (PTP-Pez; #1)	Ambion	CGAGUAGAGCUGAUACCAA
PTPN14 (PTP-Pez; #2)	Ambion	CCACGAAGUUUCGAACGGA
PTPN12 (PTPPEST; #7)	Qiagen	UUGCAGGUUAUCAGAGAUCAA
PTPN12 (PTPPEST; #8)	Qiagen	AAGCUUAAUGAGGAAAUAUCA
PTPN11 (SHP2; #5)	Qiagen	AAGGUGGUUUCAUGGACAUCU
PTPN11 (SHP2; #6)	Qiagen	CAGAAGCACAGUACCGAUUUA
PTPN1 (PTP1B; #6)	Qiagen	GUCAGUCCCUUUGACCAUA
PTPN1 (PTP1B; #7)	Qiagen	GGAGAAAGGUUCGUUAAAA
PTPRA (#5)	Qiagen	CAGAGUGAUCAUUCCAGUUAA
PTPRA (#6)	Qiagen	CCGGAGAAUGGCAGACGACAA

2.1.3 Antibodies

Antibodies to DDR1, ABL1, c-Kit, ERK, FAK, NCK (15B9), Phospho-p130Cas (Y410), Phospho-p130Cas (Y165), Phospho-ERK (T202/Y204), Phospho-Paxillin (Y118), Phospho-Src (Y416), PP2AC, PTP1B, PTP-PEST (AG10), Phospho-PTPRA (Y789), Phospho-Src (Y416), SHP2, and Alexa Fluor® 488 were from Cell Signaling Technology Inc. (Danvers, MA, USA). Antibodies to PDGFR-β, β1 Integrin (P5D2), β3 Integrin (H-96), CSK (CSK-04), CRAF, BRAF, p130Cas (C-20), Paxillin (H-114), Pez (G20) and GAPDH (V-18) were from Santa Cruz Inc. (Heidelberg, Germany). Secondary antibodies to mouse (SC-2005), goat (sc-2010) and rabbit (sc-2004) were also from Santa Cruz Inc. Antibody to p130Cas (Mouse) and FAK (Mouse) were from BD Transduction Laboratories (Oxford, UK). Antibody to Phospho-p130Cas (Y128) was from Generon (Maidenhead, UK). Antibody to ABL2 (N1N3) was from GeneTex (Irvine, USA). Antibodies to Phospho-FAK (Y861) and Phospho-FAK (Y397) were from Life Technologies Ltd. (Paisley, UK). Antibody to Phospho-Paxillin (S126) was from Abcam (Cambridge, UK). Anti-phosphoserine antibody (AB1603) was from Millipore. Please refer to **Table 2.1.3** for the dilutions of primary antibodies that were most commonly used for Western blotting. Secondary antibodies were all used at a dilution of 1:10,000.

Table 2.1.3 List of antibodies and dilutions used for Western blot. BD, BD Transduction Laboratories; CST, Cell Signaling Technology Inc.; LT, Life Technologies Ltd.; SC, Santa Cruz Inc.

TARGET	MANUFACTURER	HOST	DILUTION
ABL1	CST	Rabbit	1:1000
ABL2	GeneTex	Rabbit	1:1000
BRAF	SC	Rabbit	1:1000
c-Kit	CST	Mouse	1:1000
CRAF	SC	Rabbit	1:1000
CSK	SC	Mouse	1:1000
DDR1	CST	Rabbit	1:1000
ERK	CST	Rabbit	1:1000
ERK phospho T202/Y204	CST	Rabbit	1:1000
FAK	CST	Rabbit	1:1000
FAK	BD	Mouse	1:1000
FAK phospho-Y397	LT	Rabbit	1:1000
FAK phospho-Y861	LT	Rabbit	1:000
GAPDH	SC	Goat	1:5000
NCK1	CST	Rabbit	1:1000
p130Cas	BD	Mouse	1:5000
p130Cas	SC	Rabbit	1:1000
p130Cas phospho-Y128	Generon	Rabbit	1:1000
p130Cas phospho-Y165	CST	Rabbit	1:1000
p130Cas phospho-Y410	CST	Rabbit	1:2000
Paxillin	SC	Rabbit	1:4000
Paxillin phospho-S126	Abcam	Rabbit	1:1000
Paxillin phospho-Y118	CST	Rabbit	1:1000
PDGFRβ	CST	Rabbit	1:1000
PEZ (PTPN14)	SC	Goat	1:1000
PP2AC	CST	Rabbit	1:1000
PTP-PEST	CST	Mouse	1:1000
PTP1B	CST	Rabbit	1:1000
PTPRA phospho-Y789	CST	Rabbit	1:1000
SHP2	CST	Rabbit	1:1000
Src phospho-Y416	CST	Rabbit	1:1000
β1 Integrin	SC	Mouse	1:500
β3 Integrin	SC	Rabbit	1:1000

2.1.4 Inhibitors

The FAK inhibitor PF-573228 was purchased from Tocris Bioscience (Bristol, UK). PDGF-BB was purchased from PeproTech (London, UK). Dimethyl sulphoxide (DMSO) and lipopolysaccharide (LPS; L4130) were purchased from Sigma. The MEK1/MEK2 inhibitor U0126 (ABE2893), imatinib (Gleevec®, STI-571), and nilotinib (Tasigna ®, AMN-107) were from Source Bioscience (UK). The PP2A inhibitor okadaic acid (OA; 495609) was purchased from Millipore. The PP2A activator FTY720 (1006292) was from Cayman. The Src inhibitor PP2 (529576) was from Calbiochem.

2.1.5 Stock solutions and buffers used for immunoblotting

Cell lysis buffer per 10ml

RIPA (Sigma; 150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0)	10ml
cOmplete™ mini EDTA-free protease inhibitor tablet	1 tablet
Phosphatase inhibitor cocktail 1	10µl
Phosphatase inhibitor cocktail 2	10µl

PBST (phosphate-buffered saline with tween) per 1000ml

PBS tablets (Gibco; Phosphate 10nM, KCl 2.68nM, NaCl 140nM)	2 tablets
TWEEN®20	1000µl
Water	1000ml

NP40 Buffer per 10ml

NP40 (150mM NaCl, 50mM Tris-HCl pH8, 0.5% NP40)	10ml
cOmplete™ mini EDTA-free protease inhibitor tablet	1 tablet
Phosphatase inhibitor cocktail 1	10µl
Phosphatase inhibitor cocktail 2	10µl

Transfer Buffer per 1000ml

NuPAGE® Transfer buffer 20X	100ml
Methanol	100ml
Water	800ml

MOPS per 1000ml

NuPAGE® MOPS 20X (50 mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7)	50ml
Water	950ml

2.2 Methods

2.2.1 Routine cell culture

All tissue culture was performed in sterile conditions using biological safety class II vertical laminar flow cabinets. Cells were grown in 37°C incubators maintained at 5% CO_2. U87MG, U251MG and U118MG glioma cells were purchased from ATCC (Manassas, USA). Hepatocellular carcinoma HEPG2 cells were a gift from Professor Steve Humphries, UCL. All these cells were cultured in DMEM containing 10% (vol/vol) foetal bovine serum (FBS) supplemented with Pen/Strep (1:100). GIST882 and GIST48 gastrointestinal cells were a gift from Professor Bart Vanhaesebroeck, UCL. GIST882 and GIST48 were cultured in Ham's F-10 medium containing 10% (vol/vol) FBS, supplemented with Pen/Strep (1:100), 1.25 ml MITO+ serum extender and 7.5 mg Bovine Pituitary Extract.

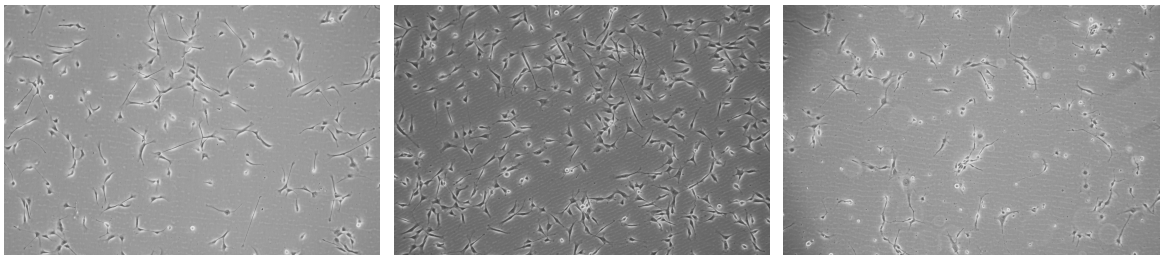
2.2.2 Culture of human glioblastoma stem cells

Human glioma stem cells (GSCs) were kindly provided by Professor Sebastian Brandner at the UCL Institute of Neurology. All patients gave informed consent before the surgical intervention. The storage of human tissue is governed by the Human Tissue Act (UK; HTA License #'s 12054). The National Hospital (part of University College London Hospitals NHS Foundation Trust) Ethics Committee has approved the use of the tissue (Ethical Approval LREC 08/0077 NHNN, HTA License #12054).

GSCs can be stimulated to proliferate by exposing them to growth factors in tissue culture to generate non-adherent spherical clusters of cells, referred to as neurospheres, that can be plated and expanded (Bez et al., 2003, Vescovi et al., 1993). The following describes how the GSCs used in this project were initially derived by Professor Brandner's lab, as described in (Jacques et al., 2010). All tumours were diagnosed as glioblastoma (WHO grade 4) by neuropathologists. The samples were taken directly from the operating theatre and placed in cold Dulbecco's Modified Eagle's Medium/Ham's F12 (DMEM/F12; Invitrogen). The samples were finely minced, erythrocytes lysed by ACK (Ammonium-Chloride-Potassium) buffer (Invitrogen) and tissue dissociated using Trypsin/EDTA. The

resulting suspension was centrifuged and pellets re-suspended in DMEM/F12 medium supplemented with B-27 (17504-044, Invitrogen), EGF (recombinant mouse, 315-09, PeproTech, 20 ng/ml), bFGF (recombinant human, 100-18B, PeproTech, 20 ng/ml) and Pen/Strep (1:100). For the first week, cells were cultured free-floating to form spheres and subsequently transferred into Laminin-coated dishes (L2020, Sigma, 0.01 mg/ml) in which they grow adherently. Cells were kept in 37°C incubators maintained at 5% CO_2.

GSCs exhibit diverse morphologies across patient samples and across clones from the same cell line (Zorniak et al., 2012). The morphology of the cells from three human-derived glioblastoma tumours used in this project is shown below (**Fig. 2.2.2**). The GSCs used here appear more astrocytic (Zorniak et al., 2012), with triangular cell bodies and multiple short projections.



NH10-1123 NH10-1124 NH12-1206

Figure 2.2.2 Morphology of three patient-derived GSCs used in this project. Cells and images were provided by Professor Brandner at the UCL Institute of Neurology. The IncuCyte ZOOM® brightfield imaging microscope was used.

2.2.3 siRNA transfection

Cells at 60% confluence were transfected with Lipofectamine 2000 using 25 nM final concentration of siRNA. **Table 2.1.2** provides details of all siRNA used for this project. Opti-MEM was used to dilute Lipofectamine 2000. Cells were transfected in 6-well tissue culture plates. The final volume used per well was 500 µl Opti-MEM and 6µl Lipofectamine 2000 with 25 nM final concentration of siRNA, added to 1.5 ml DMEM supplemented with 10% FBS. Cells were left for 48 hours following which they were shifted overnight with serum-free DMEM, before treating with drugs and harvesting.

2.2.4 Preparation of protein extracts

Cells were seeded in 6-well tissue culture plates, treated as indicated and washed twice with ice-cold PBS. Cells were harvested in RIPA or NP40 lysis buffer containing the cOmplete protease inhibitor cocktail from Roche, and phosphatase inhibitor cocktails 1 and 2 from Sigma (80 µl RIPA per well of a 6-well plate). The cells were scraped off the plate and the lysates transferred to 1.5ml centrifuge tubes. The samples were centrifuged for 15 min at 16,000 *g* at 4°C to pellet the insoluble material. The supernatant containing the protein lysate was removed and kept on ice, or at -20°C for longer-term storage.

2.2.5 Immunoprecipitation

Immunoprecipitation (IP) uses antibodies specific to a protein to remove those proteins from solution. The antibody-protein complexes were precipitated out of solution with the addition of an insoluble form of antibody binding proteins. Cells were washed with PBS, lysed in NP40 (50 mM Tris–HCl pH8, 150 mM NaCl, 0.5% NP40) containing cOmplete protease and phosphatase inhibitors and centrifuged for 15 min at 16000 *g* at 4 °C. Immune complexes were collected using antibody and rotating at 4˚C overnight. 20 µl of anti-mouse or anti-rabbit IgG agarose beads were added for an additional 1 hour. The beads were collected by centrifuging at 16000 *g* for one minute and washed three times with ice-cold lysis buffer, and once with ice-cold PBS. Samples were eluted with 20µl of 2x SDS sample

buffer and heated for 2 min at 80^{o}C. Samples were separated and immunoblotted as described in the next section. The following beads were used: Anti-Rabbit IgG (whole-molecule)-Agarose, antibody produced in goat (Sigma; A8914); Control Rabbit IgG-Agarose (Sigma; A2909), Anti-Mouse IgG (whole-molecule)-Agarose, antibody produced in goat (Sigma; A6531); Control Mouse IgG-Agarose (Sigma; A0919).

2.2.6 Immunoblot analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to separate proteins according to their molecular weight. Equal concentration of protein samples were reduced in 4x SDS-PAGE sample buffer followed by heating at 80˚C for 3 minutes. After centrifugation, protein samples were resolved on precast polyacrylamide gels (4-12% NuPAGE Bis-Tris) immersed in MOPS SDS running buffer. Pre-stained Spectra Multicolor Broad Range Protein Ladder was loaded to the first well of the gel while the protein samples were loaded to the subsequent wells. The gel was run for 45 minutes at 200 V to allow for the separation of proteins. Proteins were electrotransferred onto Invitrolon™ PVD membranes (0.45 μm pore) in Nu-PAGE transfer buffer containing 10 % methanol at room temperature for 1 hour at 30 V. Successful transfer was indicated by full transfer of the molecular weight markers onto the membrane. Membranes were blocked with 5% (wt/vol) non-fat dry milk and 0.1% (vol/vol) TWEEN®20 in phosphor-buffered saline (PBST) for 1 hr at room temperature at gentle rocking, before being probed with the primary antibody by overnight incubation at 4°C. The membranes were washed 3 times for 10 minutes each in PBST before adding appropriate horseradish peroxidise (HRP) conjugated anti-immunoglobulin G (IgG) secondary antibody diluted in 5 % milk/PBST solution for 1 hour. After secondary antibody incubation, membranes were washed and detected using the enhanced chemiluminescence (ECL) Western Blotting Detection Reagent following the manufacturer's protocol. Chemiluminescent images of immunodetected bands were recorded on Amersham Hyperfilm chemiluminescence film and developed using the Kodak® X-Omat Model 2000 processor. Immunoblots were quantified by scanning films with a calibration strip and analysed by densitometry using Image J (US National Institutes of Health; http://rsb.info.nih.gov/ij). Results, were normalised to total protein levels to account for loading errors or incomplete transfers.

2.2.7 Immunofluorescence

For immunofluorescent staining, cells were fixed in 4% paraformaldehyde (PFA) for 20 min followed by permeabilisation in 0.2% Triton X-100 for 30 min and washed in Tris Buffered Saline (TBS) with 0.1% Triton X-100. After blocking with 2% BSA/0.1% Triton X-100/TBS for 1 hour at room temperature, cells were incubated with primary antibodies overnight at 4^{o}C (dilutions shown below). Primary antibody incubation was followed by three washes with TBS with 0.1% Triton X-100 and subsequent incubation with secondary antibodies conjugated to Alexa 488 fluorescent dye for 45 minutes. Cells were washed three times and mounted on glass slides using immunofluorescence Vectashield mounting medium with DAPI. Confocal imaging was performed using an Olympus FV1000 confocal microscope. Images were obtained with a x60 magnification oil-immersion objective. The following dilutions were used for immunofluorescence: Anti-phospho FAK Y861, anti-phospho p130Cas Y410, anti-phospho Paxillin Y118, and Alexa Fluor® 488 were used at 1:200.

2.2.8 Spheroid invasion assay

Methyl cellulose was dissolved in distilled water, shaken vigorously overnight at 37^{o}C and autoclaved (stored at 4^{o}C). Cells were trypsinised and counted using the ADAM MC automated cell counter (NanoEnTek Inc., Newton, USA). A total of 5X10^{4} cells/ml were suspended in a 4:1 (vol/vol) mixture of 10% FBS in DMEM and methyl cellulose. 100µl of mixture was pipetted and plated per one well of a non-tissue culture treated u-bottom 96-well plate and incubated for 24 h (37^{o}C, 5% CO2). The next day the collagen mixture was prepared on ice containing the following per 1000µl: 700µl of collagen, 200µl of 5XDMEM and 100µl distilled water. The collagen mixture was divided into falcon tubes and supplemented with control DMSO or various inhibitors, mixed well, and left on ice. 500µl of collagen + treatment was transferred per well of a 12-well plate and left to solidify in an incubator at 37^{o}C for 1 hour. The medium in the 96-well plate containing the spheroids was carefully aspirated, making sure the spheroids were left at the bottom of the well. 100µl of the collagen mix + treatment was added to each spheroid. The tip of 1000µl tip was cut with sterilised scissors and used to pick up the spheroids with collagen mix and added to the solidified collagen in the 12-well plate. 5 spheroids per condition were added. Spheroids were left to invade for 48h following which they were fixed with 4% PFA and images were taken using a phase contrast microscope. Invasion was determined by measuring the circular area of the spheroid core and the rim of the invasion using Image J (**Fig. 2.2.8**).

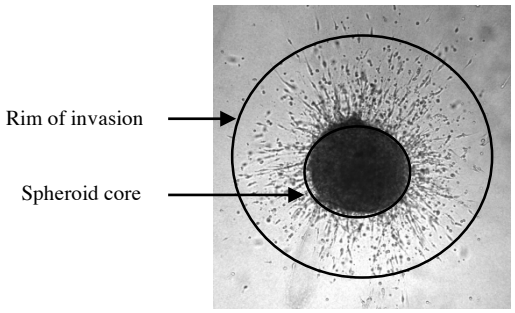


Figure 2.2.8 Example of a spheroid generated using the spheroid invasion protocol. The core and rim of invasion used for quantification are shown**.**

2.2.9 Transwell migration assay

Transwell migration assays are used for the study of motility of different types of cells. Permeable migration inserts are placed in a well of a tissue-coated plate, creating two chambers on either side of the insert. Cells placed in the top chamber can sense chemoattractant(s) placed on the other side and can diffuse across the permeable insert. Cells which migrate across can be counted to assess the effect of the chemoattractant on motility. Transwell cell culture inserts made of transparent, low-pore-density polyethylene terephthalate with an 8 μm pore size were inserted into a 24-well plate. Serum free medium supplemented with vehicle or imatinib was added to the bottom chamber, and U87 glioma cells in suspension (1.5 x 10^5 cells/well in serum free DMEM) were added to the top chamber and incubated at 37˚C for 6h. Cells that had not migrated or had only adhered to the upper side of the membrane were removed before membranes were fixed and stained with a Reastain Quik-Diff kit (IBG Immucor Ltd, West Sussex, UK) and mounted on a glass slide. Cells that had migrated to the lower side of the membrane were counted in four random fields per well at x20 magnification using an indexed eyepiece graticule.

2.2.10 Wound healing assay

The wound healing assay, also known as the scratch assay, allows for the study of two-dimensional migration *in vitro*. By creating an artificial gap (the so-called "wound" or "scratch") on a monolayer of cells, the cells on the edge of the gap will move toward the other edge to close the gap. The rate of wound closure can be calculated by capturing images at the beginning and at regular intervals thereafter (**Fig.2.2.10**).

U251MG cells were seeded to confluence in a tissue culture flat-bottom 96-well plate. Cells were shifted and treated with imatinib, nilotinib, or vehicle control. The IncuCyte™ WoundMaker™ (Essen Bioscience, UK) was used to mechanically create a homogenous 700-800 µm wide scratch in the cell monolayer evenly across all wells of the 96-well plate. The rate of migration was measured using the IncuCyte ZOOM® live cell imaging machine (Essen Bioscience, UK) by analysing the gap over time. Photos were taken every hour for 30 hours. A minimum of six separate wells were analysed per condition in each experiment.

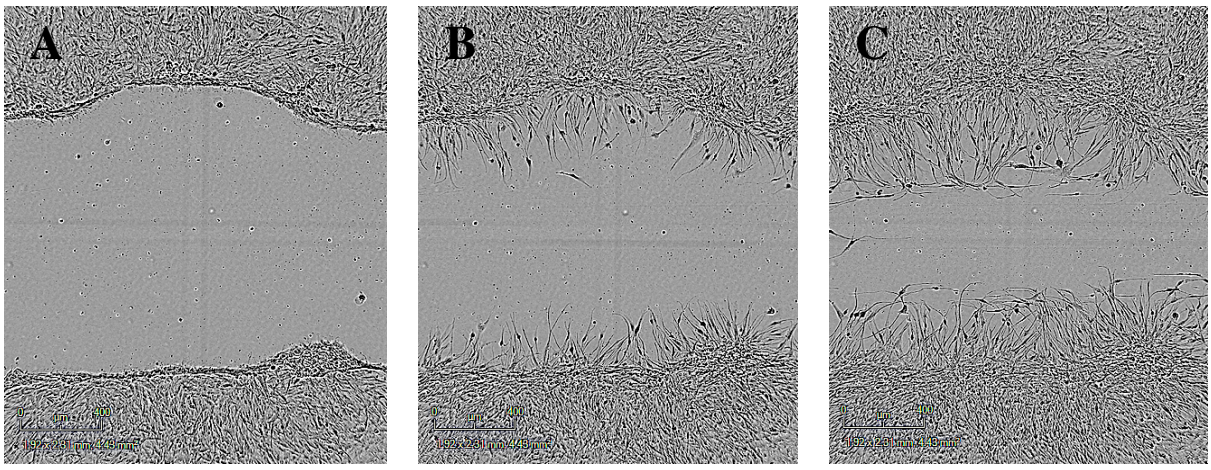


Figure 2.2.10 Example images of wound closure captured using the IncuCyte ZOOM® live cell imaging machine. U251MG cells seeded to confluence in a 96-well tissue culture-treated plate formed a monolayer. Cells were scratched and placed into the IncuCyte ZOOM®. Images were acquired at 0 h (**A**), 12 h (**B**), and 24 h (**C**).

2.2.11 Proliferation assay

Our lab has previously transfected U87MG cells with the human histone H2B gene fused to green fluorescent protein (H2B-GFP). The H2B-GFP fusion protein is incorporated into nucleosomes, the building blocks of chromatin. U87MG stably expressing H2B-GFP were seeded in 96-well plates at a density of 2,000 cells / well. Proliferation was determined by assessing the total cell fluorescence intensity per well in living cells using the IncuCyte ZOOM® for up to 96 hours.

2.2.12 Statistical analysis

For the Western blot results, data was obtained from at least three separate experiments and representative blots are shown to present protein expression. For the spheroid assay results, data was obtained from three separate experiments, with at least three different spheroids measured per condition. Statistical analysis was performed using Prism (GraphPad) on normalised data by one- or two-way analysis of variance (ANOVA) followed by the Student Newman Keuls test for multiple comparisons used to find differences. The level of accepted statistical significance was $P<0.05$. The data displayed on the graphs are means, with error bars representing the standard error of the mean (SEM). $P<0.05$ is denoted with * and $P<0.01$ is denoted with **.

3 Imatinib and nilotinib stimulate tyrosine phosphorylation and promote tumour cell migration in human GBM cells

3.1 Imatinib and nilotinib treatment of human GBM cells leads to increased p130Cas, FAK and PXN tyrosine phosphorylation

Access to readily available cell models is critical for the study of GBM. U87MG is a well-characterised and commonly studied GBM cell line that has been used for research for decades and is included in over 1900 publications. The U87MG cell line was initially derived from a primary brain tumour from a 44-year-old Caucasian woman. The cells are easy to procure and to maintain for multiple passages (Clark et al., 2010, Xiang et al., 2015, Xie et al., 2015). U87MG cells used in this project were previously purchased by our lab. The cells are well characterised and are known to express both PDGF-A and PDGF-B, and high levels of PGFRα and β receptor. They have mutations in the p14, p16 and PTEN tumour suppressors (Shamah et al., 1993, Kilic et al., 2000, Ishii et al., 1999).

Tyrosine phosphorylation of p130Cas at Y410, FAK at Y861 and PXN at Y118 play important roles in cell migration and invasion. Tyrosine residue Y410 of p130Cas is phosphorylated in response to PDGF- and HGF-mediated migration of U87MG glioma cells (Evans et al., 2011). Src phosphorylation of Y861 enhances FAK binding to p130Cas (Mitra et al., 2005). Phosphorylation of PXN at tyrosine residue Y118 is required for the formation

of focal adhesions and for optimum motility. Tyrosine phosphorylation at residue Y118 (and Y31) generates a functional SH2-binding site for members of the Crk family of SH2-SH3 adaptor proteins (Petit et al., 2000).

U87MG cells were treated with imatinib or nilotinib to investigate their effects on key signalling proteins required for tumour cell motility and invasion, p130Cas, FAK and PXN. Tyrosine residues Y410, Y861 and Y118 were used as readouts of activation of p130Cas, FAK and PXN, respectively. Treatment with 10 μM imatinib or 10 μM nilotinib caused a striking increase in tyrosine phosphorylation of p130Cas, FAK and PXN (**Fig. 3.1.1**). The effects observed were dose-dependent, with a significant increase detected at 1 μM and a maximal response at 10 μM for both imatinib and nilotinib (**Fig. 3.1.2**). The effects of imatinib and nilotinib on tyrosine phosphorylation of p130Cas, FAK and PXN are rapid and sustained with a significant increase observed at 10 min, reaching a maximum at 30 min, which are maintained for up to 1 hour (**Fig. 3.1.3**). After 4 hours, p130Cas tyrosine phosphorylation declined to near basal level, whereas FAK and PXN tyrosine phosphorylation remained strongly elevated.

Imatinib and nilotinib were also found to increase tyrosine phosphorylation of additional p130Cas residues. Phosphorylation of p130Cas at tyrosine residue Y128 is critical for migration of colorectal cancer cells (Zhang et al., 2013) and phosphorylation at Y165 promotes p130Cas association to FAK in cortical neurons (Liu et al., 2007a). Treatment with imatinib and nilotinib led to an increase in phosphorylation of tyrosine residues Y128 and Y165 (**Appendix Fig. A1A**). One of the earliest steps of the signalling transduction pathway mediating motility is the autophosphorylation of FAK at tyrosine residue Y397 (Sharma and Mayer, 2008). Treatment with imatinib or nilotinib also led to an increase in tyrosine phosphorylation of FAK at residue Y397 (**Appendix Fig. A1B**).

Immunofluorescence confocal microscopy was performed in order to examine the effect of imatinib and nilotinib treatment on the localisation of the phosphorylated motility-related proteins p130Cas, FAK and PXN. Our lab previously generated U87MG cells with fluorescently labelled actin cytoskeleton that stably express mCherry-LifeAct (Riedl et al., 2008). To confirm that imatinib and nilotinib cause similar effects in the U87MG LifeAct cells, the outcome of drug treatment on tyrosine phosphorylation was examined. Imatinib and nilotinib treatment of LifeAct U87MG cells induced similar increases in tyrosine phosphorylation of Y410, Y861, and Y118 (**Fig. 3.1.4**), confirming that the LifeAct cells

behave similarly to the parental cell line. Subsequent immunofluorescence revealed that the levels of phosphorylated p130Cas (Tyr 410) increased in both imatinib and nilotinib treated cells after 20 min. A striking redistribution of phoso-p130Cas to the cell membrane was also observed, localising along what appear to be membrane ruffles (**Fig. 3.1.5**). Similar increases and redistribution were observed for phospho-FAK (Y861) (**Fig. 3.1.6**) and phospho-PXN (Y118) (**Fig. 3.1.7**).

To confirm the effects of imatinib and nilotinib on tyrosine phosphorylation in GBM, the effect of drug treatment on additional well-established glioma cell lines was examined. U251MG derived from a 75-year-old male, and U118MG derived from a 50-year-old male are GBM cell lines characterised by mutations in PTEN, p53, p14 and p16 tumour suppressors (Ishii et al., 1999). 20-minute treatment with 10 μM nilotinib caused a significant increase in phosphorylation of p130Cas. Treatment with 1 μM or 10 μM nilotinib also led to a significant increase in phosphorylation of FAK and PXN. Treatment with 10 μM imatinib led to a significant increase in FAK phosphorylation (**Fig. 3.1.8**). Imatinib and nilotinib treatment caused similar increases in tyrosine phosphorylation in U118MG cells. 20-minute nilotinib treatment at 10 μM led to a significant increase in phosphorylation of all three proteins. Treatment with 10 μM imatinib led to a significant increase in phosphorylation of PXN (**Fig. 3.1.9**). The results from additional glioma cell lines support the initial findings in U87MG that two well-established TKIs induce tyrosine phosphorylation of key motility-related proteins in glioma cells.

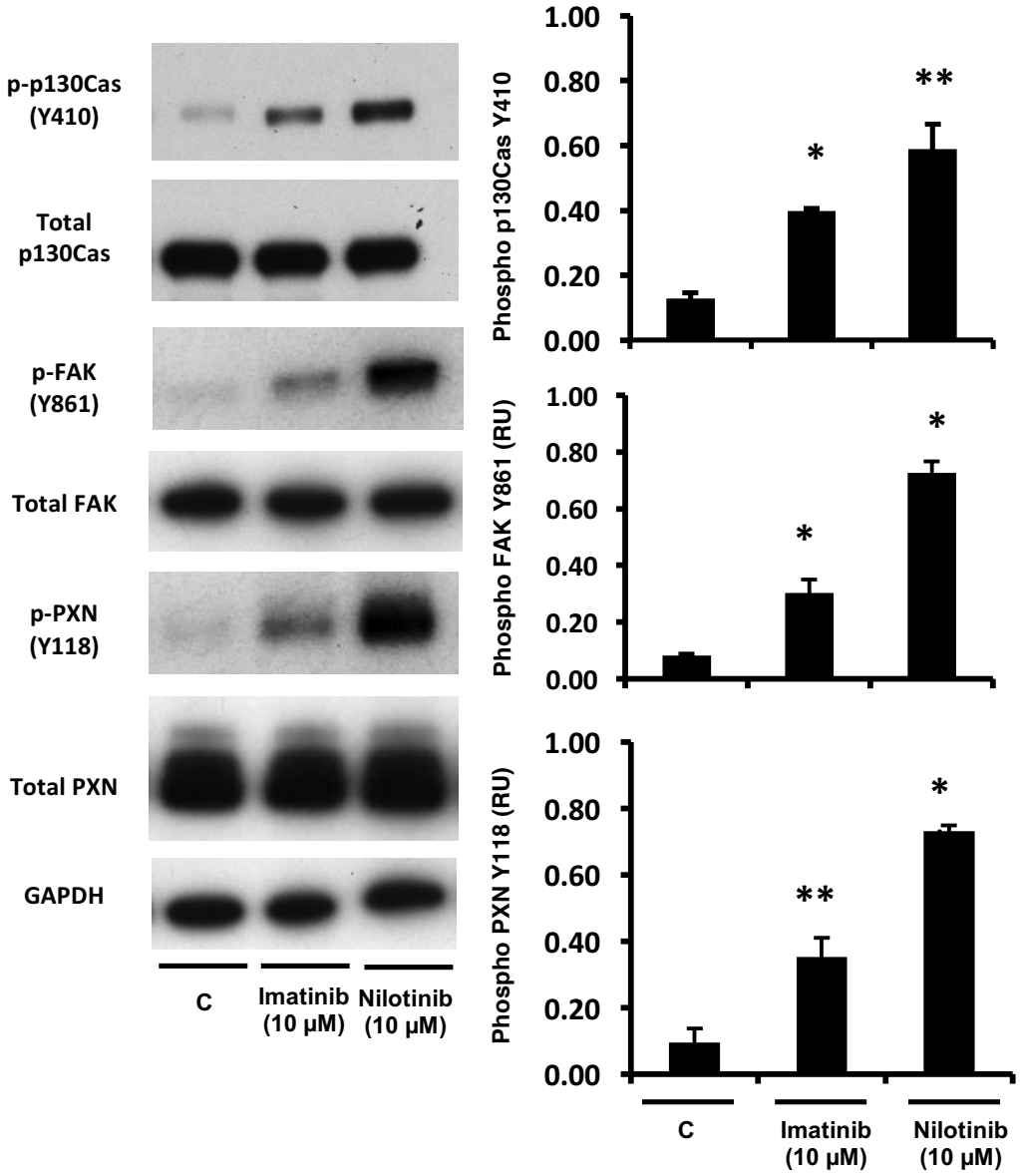


Figure 3.1.1 Imatinib and nilotinib treatment of human U87MG GBM cells leads to increased p130Cas, FAK and PXN tyrosine phosphorylation. U87MG cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with either SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Quantification of tyrosine phosphorylation shown here and in all subsequent figures was performed by densitometry using Image J. In each panel, data from four independent experiments are presented as phosphorylation relative units (RU) (means +/- s.e.m.) normalised to total protein levels; *$p<0.05$, **$p<0.01$ compared to vehicle control (C).

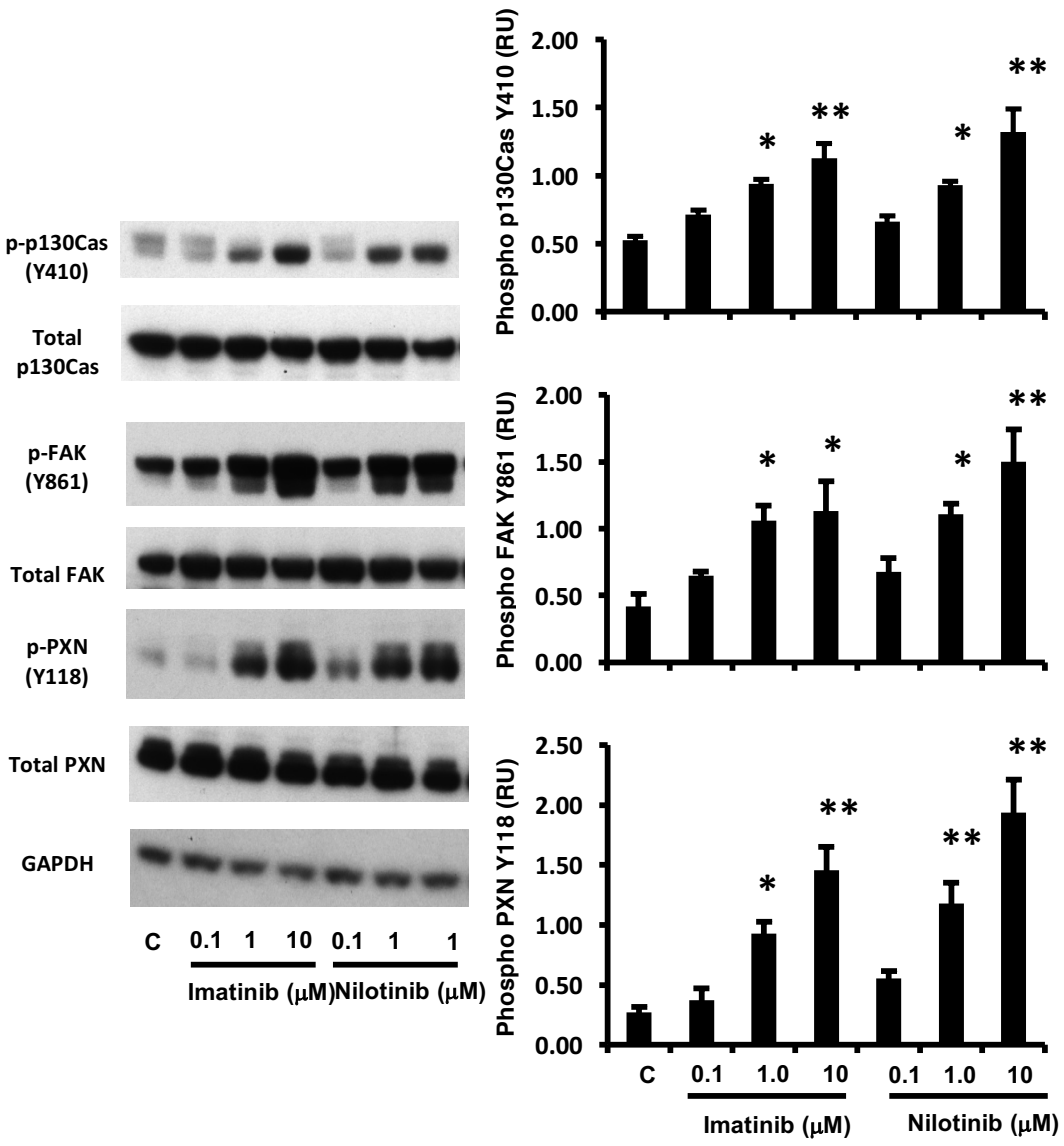


Figure 3.1.2 Imatinib and nilotinib induce tyrosine phosphorylation in a dose-dependent manner. U87MG cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with either SFM & DMSO vehicle control (C), imatinib or nilotinib at indicated concentrations for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Data from three independent experiments are presented as phosphorylation relative units (RU) (means +/- s.e.m.) normalised to total protein levels; *p<0.05, **p<0.01 compared to vehicle control (C).

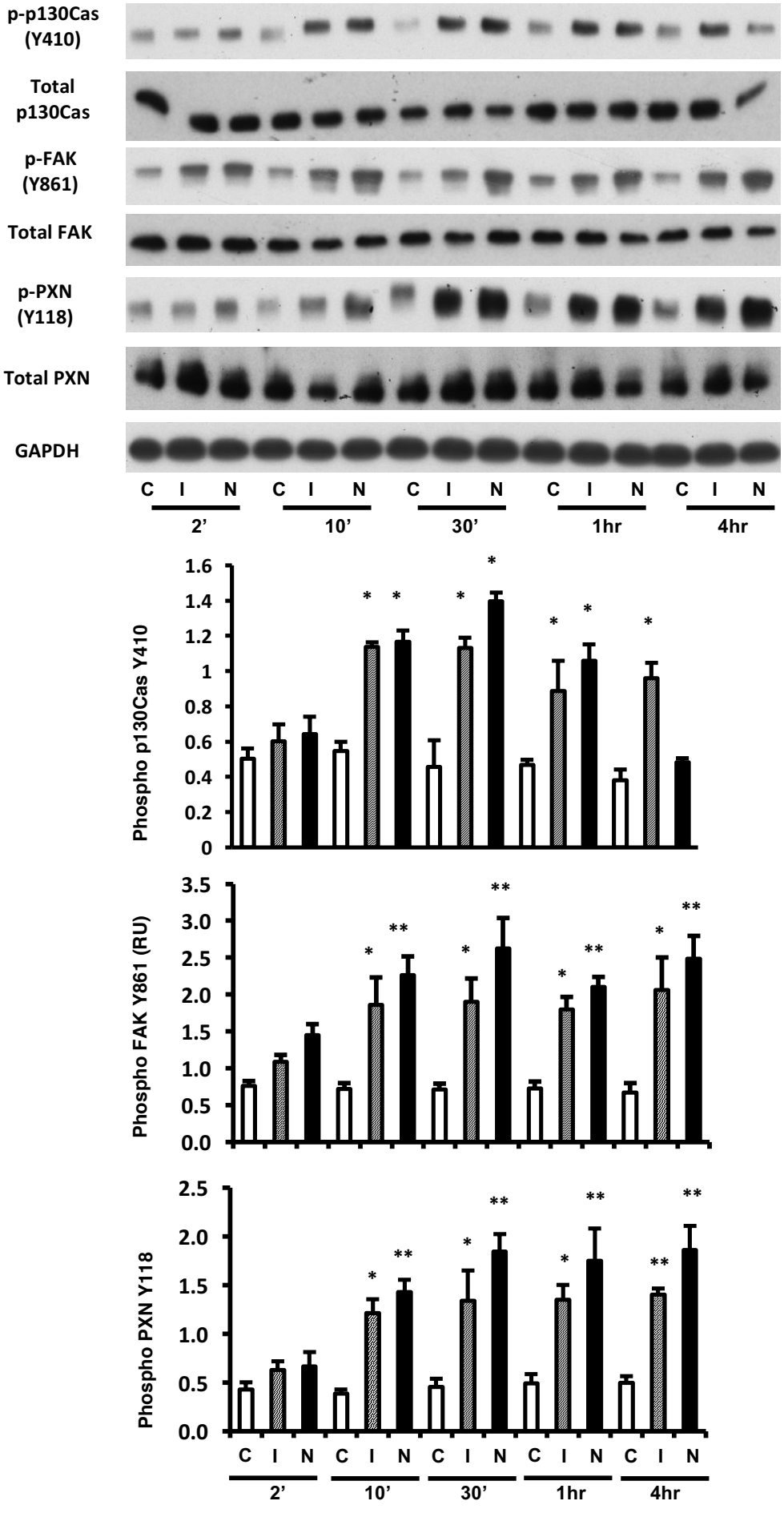

p-p130Cas (Y410)
Total p130Cas
p-FAK (Y861)
Total FAK
p-PXN (Y118)
Total PXN
GAPDH
C I N C I N C I N C I N C I N
2’ 10’ 30’ 1hr 4hr
Phospho p130Cas Y410
Phospho FAK Y861 (RU)
Phospho PXN Y118
C I N C I N C I N C I N C I N
2’ 10’ 30’ 1hr 4hr

Figure 3.1.3 Time course of imatinib- and nilotinib-induced tyrosine phosphorylation. U87MG cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with either SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for the indicated amount of time. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Data from three independent experiments are presented as phosphorylation relative units (RU) (means +/- s.e.m.) normalised to total protein levels; *p<0.05, **p<0.01 compared to vehicle control (C).

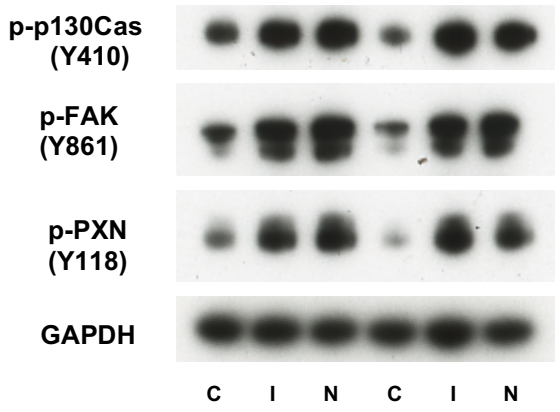


Figure 3.1.4 Imatinib and nilotinib treatment of human LifeAct GBM cells leads to increased p130Cas, FAK and PXN tyrosine phosphorylation. LifeAct GBM cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with either SFM & DMSO vehicle control (C), or 10 μM imatinib (I) or 10 μM nilotinib (N) for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies.

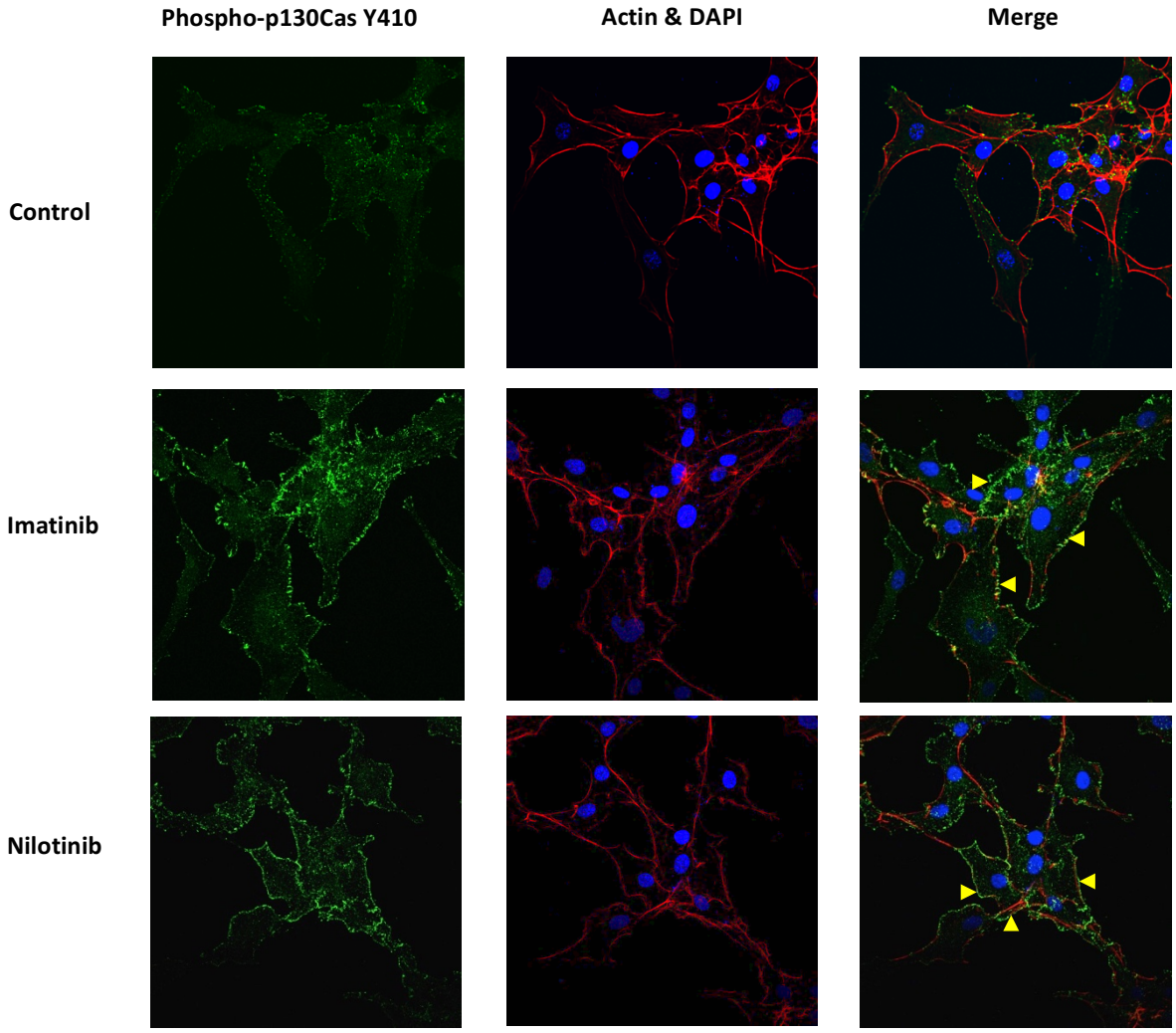


Figure 3.1.5 Imatinib and nilotinib treatment leads to a rapid increase of phospho-p130Cas at the cell membrane. Immunofluorescence confocal microscopy was performed on U87MG cells stably transfected with LifeAct®–TagRFP. Cells were seeded on glass cover slips and incubated in SFM for ~18h prior to treatment with vehicle control, or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Phosphorylated p130Cas (Y410) staining is in green, actin in red, and DAPI in purple. Arrows point to areas of increased phosphorylated p130Cas (Y410) localisation to the membrane upon treatment.

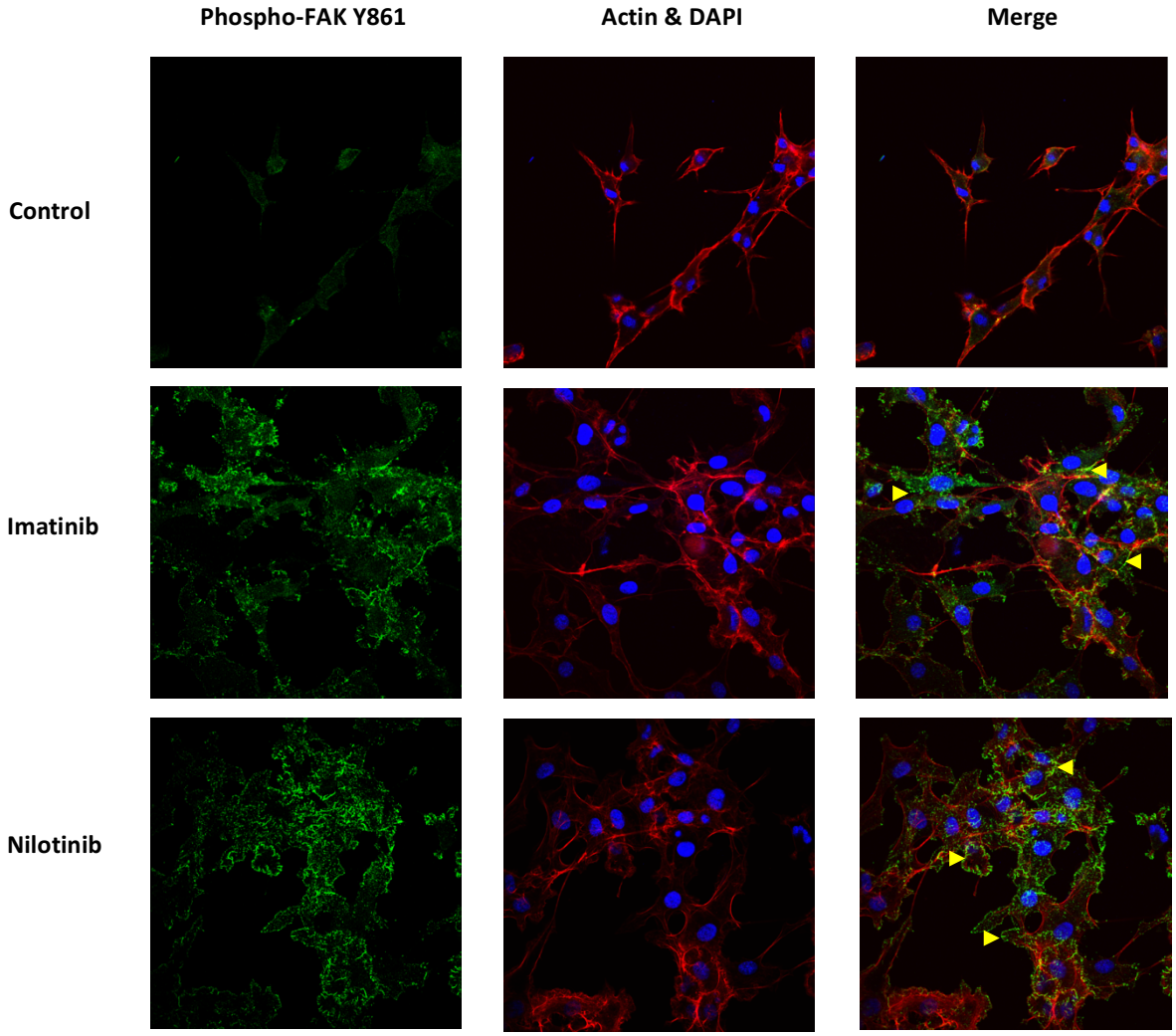


Figure 3.1.6 Imatinib and nilotinib treatment leads to a rapid increase of phospho-FAK at the cell membrane. U87MG LifeAct cells were seeded on glass cover slips and incubated in SFM for ~18h prior to treatment with vehicle control, or 10 µM imatinib or 10 µM nilotinib for 20 minutes. Phosphorylated FAK (Y861) staining is in green, actin in red, and DAPI in purple. Arrows point to areas of increased phosphorylated FAK (Y861) localisation to the membrane upon treatment.

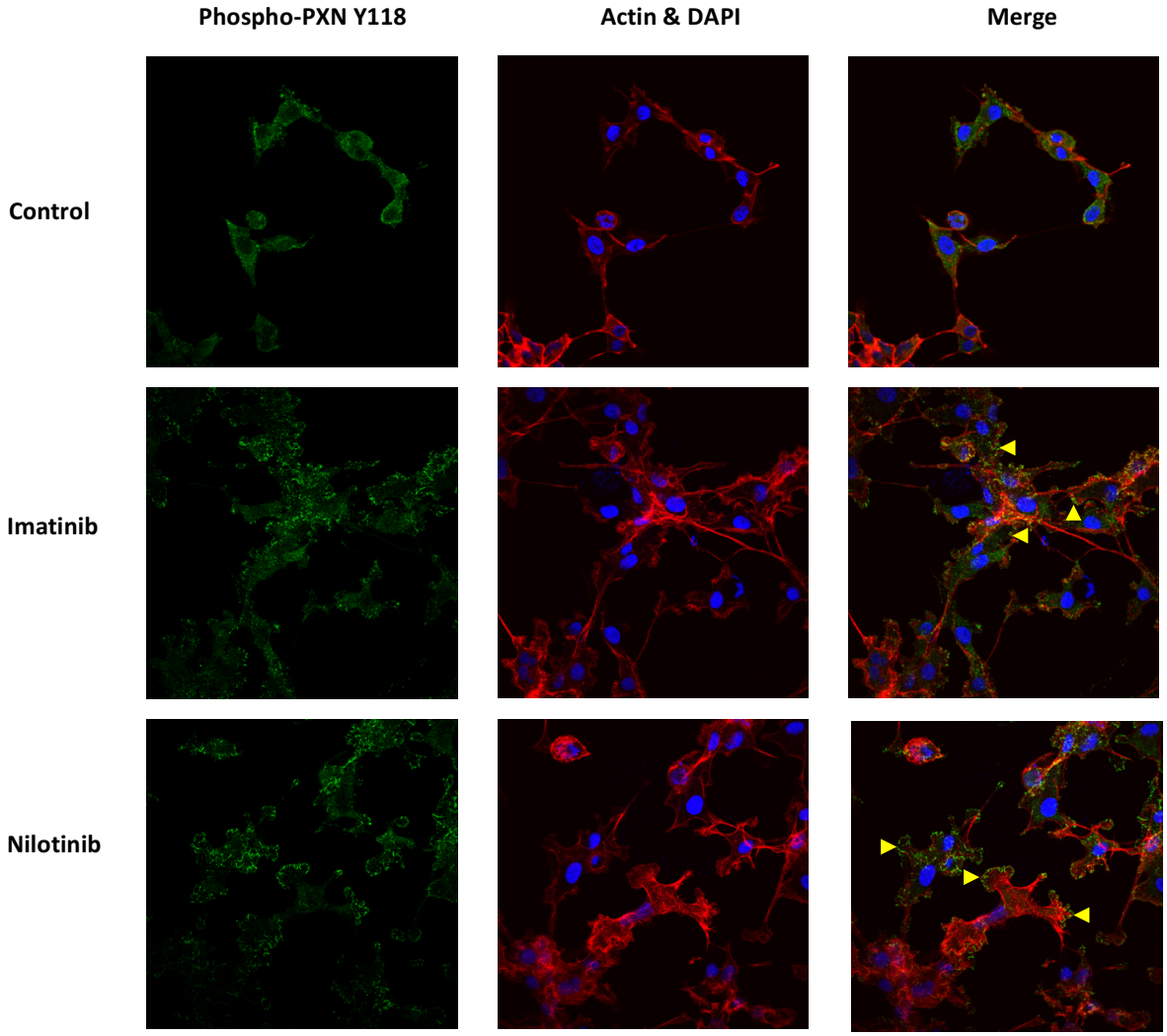


Figure 3.1.7 Imatinib and nilotinib treatment leads to a rapid increase of phospho-PXN at the cell membrane. U87MG LifeAct cells were seeded on glass cover slips and incubated in SFM for ~18h prior to treatment with vehicle control, or 10 µM imatinib or 10 µM nilotinib for 20 minutes. Phosphorylated PXN (Y118) staining is in green, actin in red, and DAPI in purple. Arrows point to areas of increased phosphorylated PXN (Y118) localisation to the membrane upon treatment.

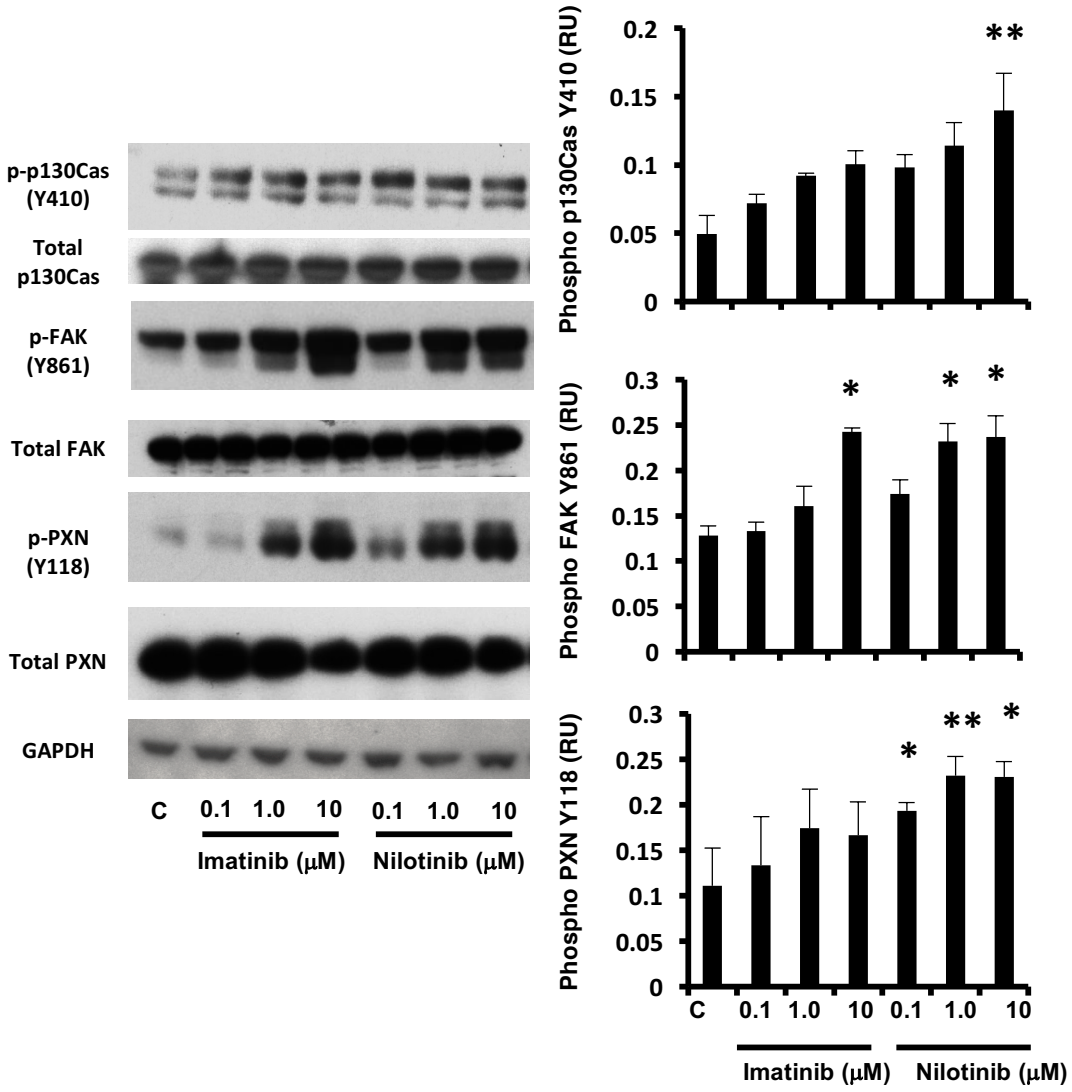


Figure 3.1.8 Imatinib and nilotinib treatment of human U251MG GBM cells leads to increased p130Cas, FAK and PXN tyrosine phosphorylation. U251MG cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with vehicle control (C), or imatinib and nilotinib at specified concentrations for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Data from three independent experiments are presented as phosphorylation relative units (RU) (means +/- s.e.m.) normalised to total protein levels; *p<0.05, **p<0.01 compared to vehicle control (C).

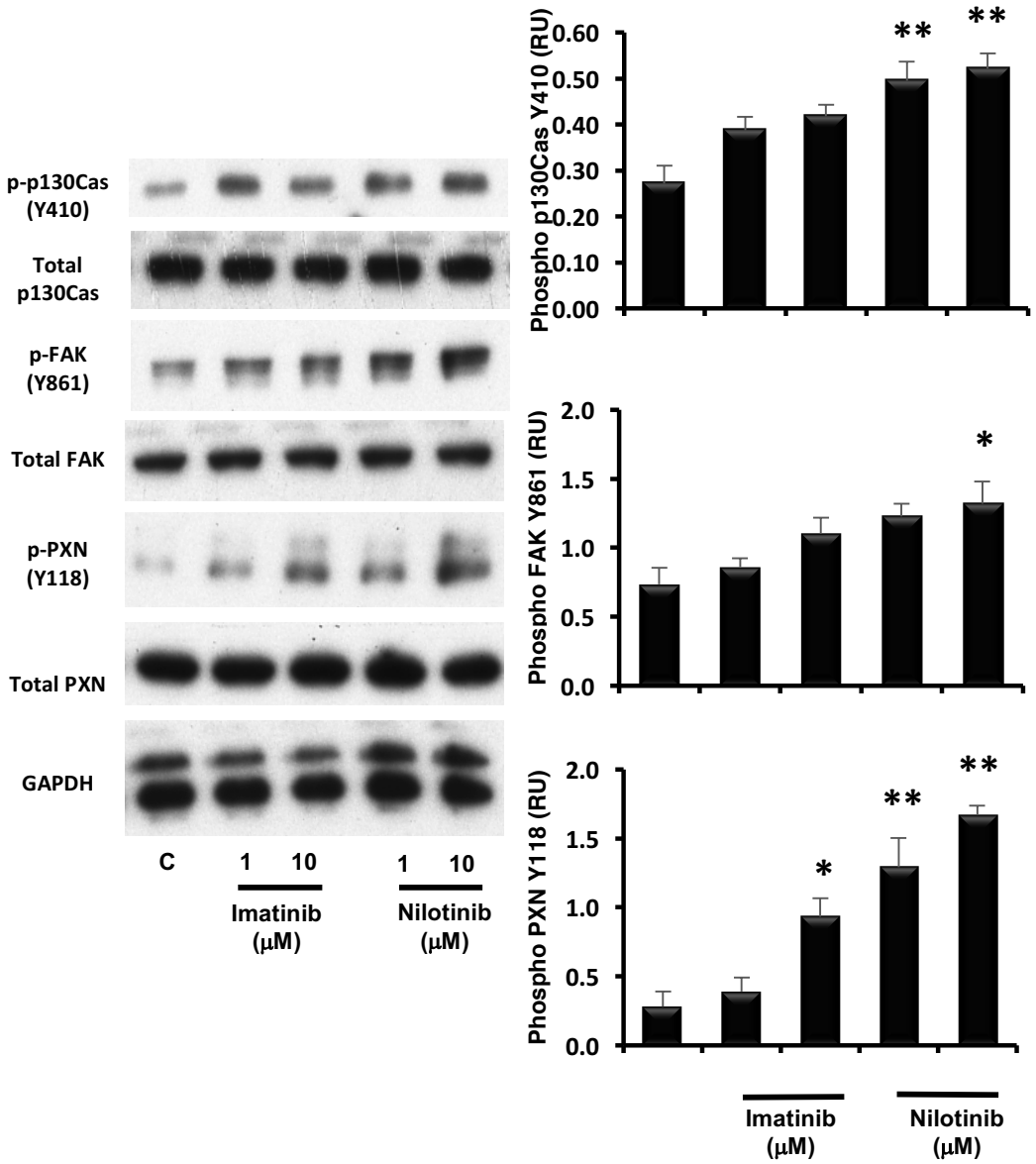


Figure 3.1.9 Imatinib and nilotinib treatment of human U118MG GBM cells leads to increased p130Cas, FAK and PXN tyrosine phosphorylation. U118MG cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with vehicle control (C), or imatinib and nilotinib at specified concentrations for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Data from three independent experiments are presented as phosphorylation relative units (RU) (means +/- s.e.m.) normalised to total protein levels; *$p<0.05$, **$p<0.01$ compared to vehicle control (C).

3.2 Imatinib and nilotinib treatment of human GBM cells leads to enhanced two-dimensional and three-dimensional migration

Signalling via p130Cas, FAK and PXN plays a critical role in the regulation of motility, and tyrosine phosphorylation at Y410, Y861 and Y118, respectively, are indicators of protein activation. Given the implication of p130Cas, FAK, and PXN in migration, the effects of imatinib and nilotinib treatment on glioma cell motility was examined. It was predicted that imatinib and nilotinib would stimulate migration. Assays for examining both two-dimensional (2D) wound healing and three-dimensional (3D) migration were employed.

The wound healing assay (also known as the scratch assay) allows for the study of 2D cell migration *in vitro*. The assay involves creating an artificial gap, the so-called "scratch", on a confluent cell monolayer. The cells on either side of the outer edge of the scratch will migrate towards the opposite edge until new cell-cell contacts are made in order to close the gap. The rate of closure is measured by taking images at the start and at regular intervals thereafter.

U87MG grow on top of each other and do not form a clear monolayer. By contrast, U251MG cells form a monolayer (**Fig. 2.2.10**) and were used for the wound-healing assay. The assay revealed that either 10 μM imatinib or 1 μM nilotinib treatment results in increased rates of wound closure compared to control vehicle-treated cells (**Fig. 3.2.1**). Nilotinib was used at 1 μM because 10 μM nilotinib precipitated out of solution after several hours.

The transwell chemotactic migration assay is a useful tool for looking at the effect of a drug on chemotaxis, which refers to the migration of cells in response to a stimulus. Permeable inserts placed in a 6-well plate create two chambers on either side of the insert. Serum free medium supplemented with or without drug can be added to the bottom chamber, and U87MG cells can be suspended in medium in the top chamber. Cells that diffuse across the insert are counted to assess the effect of the drug on migration. Transwell migration assays performed by my supervisor, Dr. Paul Frankel, show that imatinib acts as a chemo-attractant and stimulates migration of U87MG cells (**Appendix Fig. A2**).

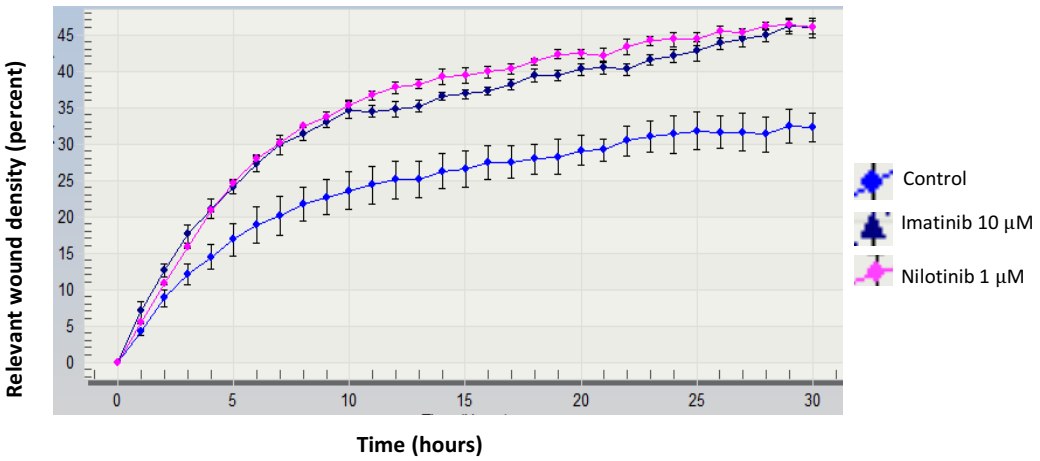
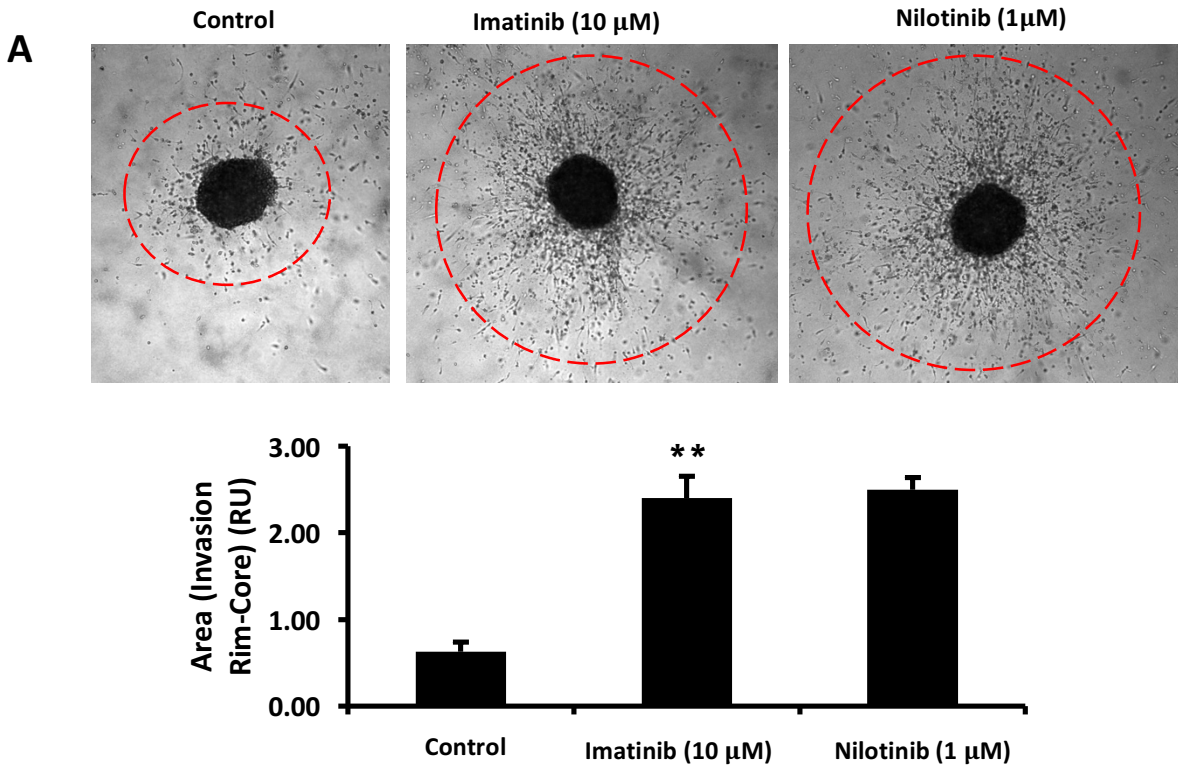
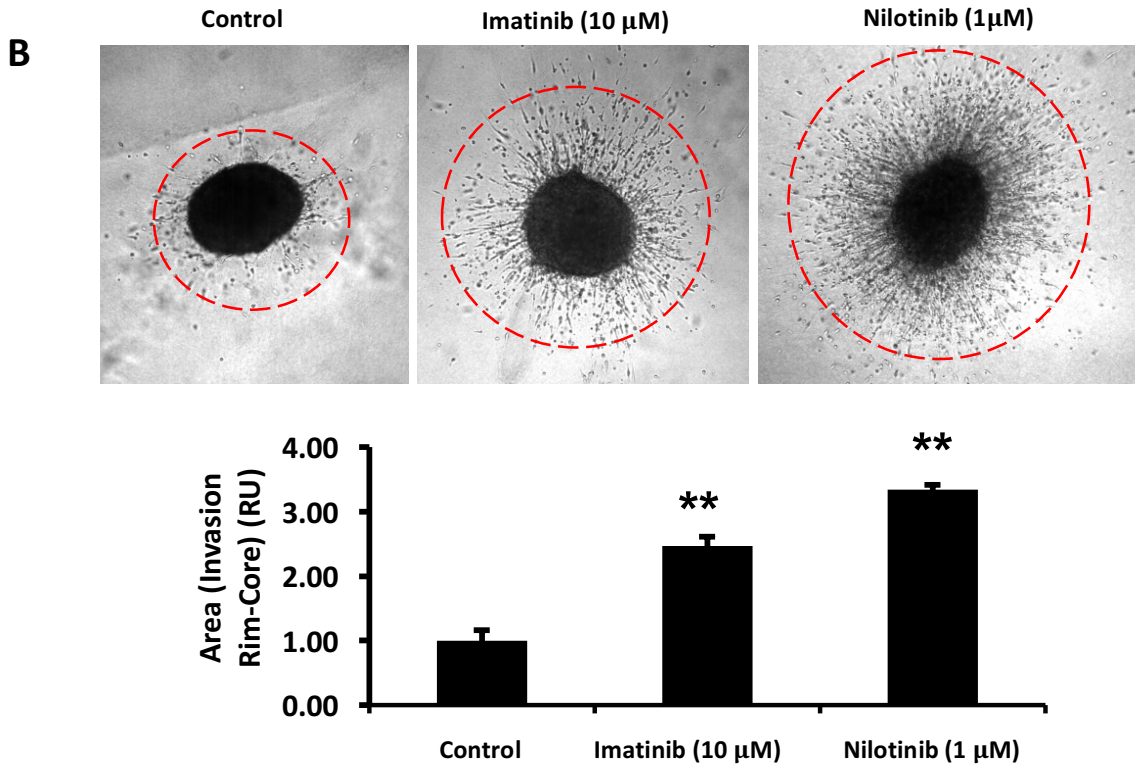


Figure 3.2.1 Imatinib and nilotinib treatment of U251MG results in enhanced rates of wound closure. U251MG cells were grown to confluence in a 96-well plate and a uniform scratch was made along the centre of each well. Cells were washed with SFM and incubated with SFM containing either DMSO control, 10 µM imatinib, or 1 µM nilotinib. Rate of wound closure was monitored in an IncuCyte ZOOM® with images captured every hour for a total of 30 hours. Data is representative of *n*=3 experiments.

Invasion of tumour cells into surrounding healthy tissue is a key hallmark of malignant tumours. GBM is a particularly malignant type of cancer with high invasive capability. The radial tumour spheroid invasion assay enables a rapid and reproducible method for investigating three-dimensional migration *in vitro*. Spheroids from U87MG, U251MG and U118MG GBM cell lines were generated and embedded in collagen plugs supplemented with serum free medium containing either vehicle control, 10 μM imatinib or 1 μM nilotinib. Treatment with either imatinib or nilotinib alone for 48 hours resulted in a striking increase in radial invasion compared to the vehicle control spheroids (**Fig. 3.2.2A–C**).

A proliferation assay was performed to confirm that the observed effects of imatinib and nilotinib on motility are not a result of enhanced proliferation. Proliferation can be assessed by labelling chromosomes in living cells with a fluorescent tag attached to a histone gene. As cells divide and proliferate the amount of fluorescent signal increases and this can be quantified using the IncuCyte ZOOM®. The effect of imatinib and nilotinib on cell proliferation was assessed by using U87MG stably expressing the histone H2B gene fused to green fluorescent protein (GFP) that were previously generated in our lab. Treatment with 10 μM imatinib or 1 μM nilotinib had no significant effect on cell proliferation (**Fig. 3.2.3**).


A
Control
Imatinib (10 µM)
Nilotinib (1µM)
Area (Invasion Rim-Core) (RU)
3.00
2.00
1.00
0.00
**
Control
Imatinib (10 µM)
Nilotinib (1 µM)


B
Control
Imatinib (10 µM)
Nilotinib (1µM)
Area (Invasion Rim-Core) (RU)
4.00
3.00
2.00
1.00
0.00
**
**
Control
Imatinib (10 µM)
Nilotinib (1 µM)

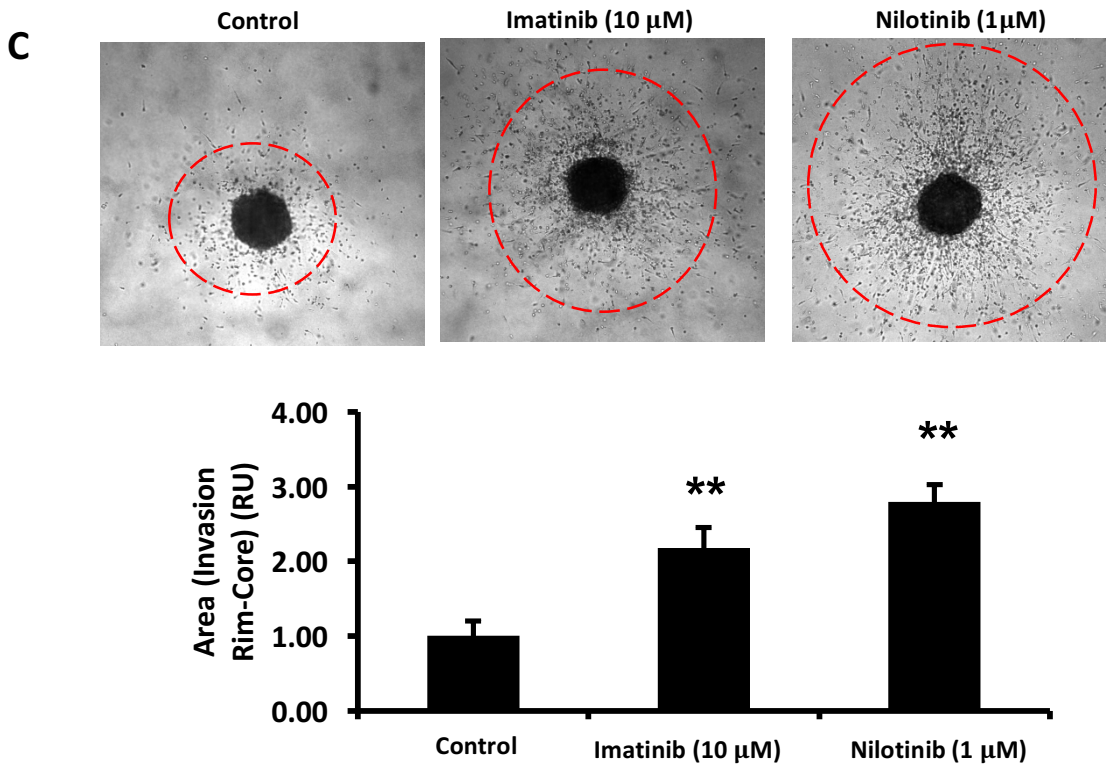


Figure 3.2.2 Imatinib and nilotinib treatment of human GBM cells leads to increased 3D radial invasion. (**A**) U87MG. (**B**) U251MG. (**C**) U118MG. (**A - C**) Equal amounts of cells were used to generate spheroids as described in Materials and Methods. 24 hours after spheroid production, spheroids were imbedded in a collagen gel and incubated in serum free medium supplemented with vehicle (control), 10 µM imatinib or 1 µM nilotinib. Spheroids were fixed in 4% paraformaldehyde (PFA) and invasion was determined by measuring the area corresponding to the invasion rim minus the area of the core for at least 3 different spheroids per condition. Data from three independent experiments are presented as relative area units (RU) (means +/- s.e.m.); **p<0.01 compared to control.

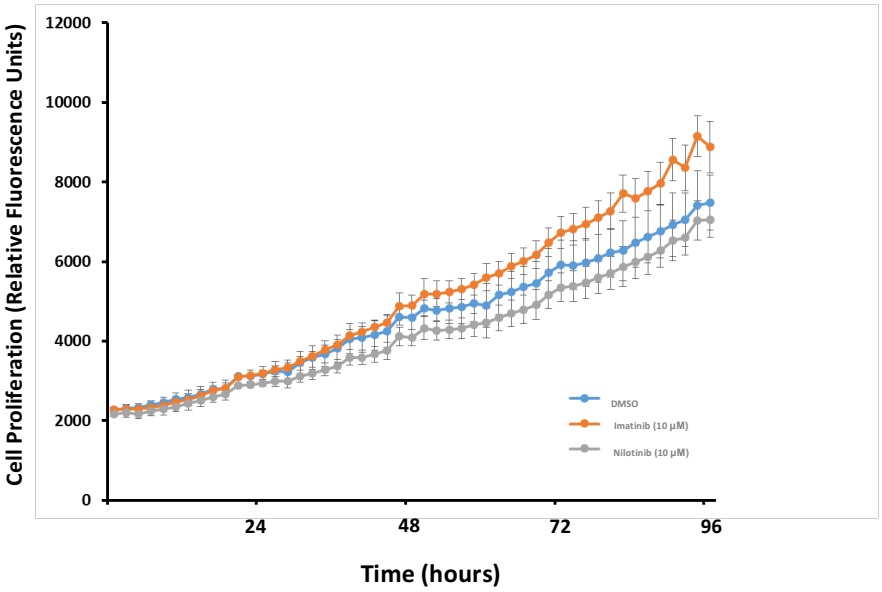


Figure 3.2.3 Imatinib and nilotinib treatment does not affect U87MG cell proliferation. Proliferation was measured using U87MG cells stably expressing H2B-GFP. Values are means from 5 replicate wells per experiment and 5 independent experiments (n=5) ± s.e.m, expressed as the relative fluorescence intensity per well.

3.3 Discussion

The effects of two tyrosine kinase inhibitors, imatinib and nilotinib, on tyrosine phosphorylation were examined, as well as their effect on glioma cell migration. Findings presented here show that treatment of multiple human glioma cell lines with imatinib or nilotinib produces a rapid and significant increase in tyrosine phosphorylation of p130Cas, FAK and PXN at residues implicated in the signalling pathways governing motility.

The peak plasma/serum concentrations of imatinib and nilotinib are approximately 4.6 μM (Druker et al., 2001) and 3.6 μM (Weisberg et al., 2007), respectively. Data presented here indicate that observed effects occur at clinically relevant concentrations. The finding that imatinib and nilotinib treatment stimulates tyrosine phosphorylation as early as 10 minutes indicates that this is an immediate response of a direct drug effect on signalling components distal to p130Cas, FAK and PXN pathways, and not the result of long-term effects such as altered gene expression. Localisation of tyrosine phosphorylated p130Cas, FAK and PXN to the cell membrane is also rapid, occurring after 20 minutes of drug stimulation.

p130Cas, FAK and PXN are important signalling nodes involved in signal transduction pathways that result in enhanced motility, as outlined in the introduction (Barrett et al., 2013, Parsons, 2003). Functional experiments were carried out to investigate the effect of imatinib and nilotinib on motility. Findings show that, consistent with increases in tyrosine phosphorylation and localisation of phosphorylated proteins to the membrane, imatinib and nilotinib strikingly increase motility in 2D and 3D models using multiple glioma cell lines. The effects appear to be specific to cell motility because proliferation was unaffected by drug treatment.

Given that tyrosine phosphorylation of p130Cas at tyrosine residues Y128 and Y165 is associated to motility, the identification that imatinib and nilotinib stimulate phosphorylation at these sites support the functional data showing that both TKIs promote migration. FAK is activated and undergoes autophosphorylation at tyrosine residue Y397 in response to integrin receptor activation (Sharma and Mayer, 2008). The finding that imatinib and nilotinib treatment leads to increased phosphorylation at Y397 further supports their ability to stimulate the motility pathway and promote migration.

GBM is a highly malignant tumour that is dependent on augmented tumour cell motility. Results provide evidence of unexpected off-target effects of the use of imatinib and nilotinib treatment on GBM cell lines. This suggests a potential adverse effect of imatinib and nilotinib treatment on tumour progression, which will be further discussed in Chapter 7. Imatinib has previously failed clinical trials for the treatment of GBM, where it shows no significant inhibition of tumour growth or extension of survival (Wen et al., 2006). A previous study found that imatinib inhibited the growth of U87MG cells *in vitro* and *in vivo* in an intracranial mouse model of GBM generated by injecting U87MG cells into mice (Kilic et al., 2000). Findings here, however, present important and unforeseen adverse effects of imatinib and nilotinib on tumour cell motility, which could significantly contribute to the lack of clinical efficacy observed in these trials. Results point out the importance of screening imatinib, nilotinib, and other TKIs of interest, on multiple modes of cell motility and on different types of cancers for which they are being investigated.

4 Investigating the mechanism of imatinib and nilotinib effects in GBM cells

The molecular mechanism of action of imatinib on glioma cells is poorly understood, and the reason for its failure in the clinic remains unclear. Imatinib and nilotinib are reported to cause activation of intracellular kinases including PI3K, Akt and ERK (Fenouille et al., 2010, Mokhtari et al., 2013, Packer et al., 2011, Dong et al., 2011). The delineation of signalling pathway(s) affected by drug treatment in glioma may help to explain not only why it failed in GBM trials, but also to identify novel targets for the improvement of clinical efficacy.

Acquired resistance is common in patients receiving TKIs for the treatment of cancers. This occurs over months or years as cells accumulate mutations in the kinase domain, or reduce their dependency on the target kinase via the activation of other kinases or signalling pathways. It is critical to develop synergistic inhibitor combinations, with the simultaneous inhibition of multiple targets, either one or more kinase and / or downstream signalling effectors. The identification of novel pathways being activated by imatinib and nilotinib could help identify new targets for combinational treatment. To start with, I wanted to investigate whether one or more of the known targets that imatinib and nilotinib bind to with high specificity could be mediating observed drug effects. The known targets that imatinib and nilotinib bind to with high specificity are ABL1, ABL2, PDGFR, DDR1, and c-Kit (Hantschel et al., 2008, Deininger et al., 2005).

4.1 Evaluating the role of the major targets of imatinib and nilotinib in mediating the observed increases in phosphorylation of p130Cas, FAK and PXN

Imatinib and nilotinib are both ABL1 and ABL2 (also called ARG) kinase inhibitors. Furthermore, ABL has been reported to mediate p130Cas coupling to adaptor protein Crk, an important interaction for downstream signalling mediating motility (**Fig. 1.2**) (Barrett et al., 2013). It was therefore reasoned that ABL1 and/or ABL2 could be mediating the imatinib- and nilotinib-induced stimulation of phosphorylation and migration.

U87MG cells were treated with siRNA to ABL1 and ABL2 either individually or in combination, and subsequently treated with 10 μM imatinib, 10 μM nilotinib or vehicle control (**Fig. 4.1.1**). A reduction in total levels of p130Cas and PXN make it difficult to evaluate the effect of the knockdown. Nevertheless, densitometric analysis of the data revealed that there is no significant effect of knockdown on imatinib- and nilotinib-induced tyrosine phosphorylation of p130Cas and PXN. However, knockdown has a significant effect on drug-induced tyrosine phosphorylation of FAK. Interestingly no decrease in basal phosphorylation of FAK in the control treated cells was detected.

Imatinib and nilotinib also inhibit three additional tyrosine kinases at physiologically relevant concentrations: the platelet derived growth factor receptor (PDGFR), the stem cell growth factor receptor (c-Kit), and discoidin domain receptor tyrosine kinase 1 (DDR1) (Hantschel et al., 2008). However, silencing of PDGFRβ and DDR1 had no effect on increased tyrosine phosphorylation in response to imatinib and nilotinib (**Fig. 4.1.2 A&B**). c-Kit was not detected in U87MG cells, whereas we could readily detect c-Kit in human coronary artery smooth muscle cells (HCASMCs) (**Fig. 4.1.2 C**). This indicates that c-Kit is not significantly expressed in U87MG cells and so cannot be mediating the imatinib- and nilotinib-induced effects.

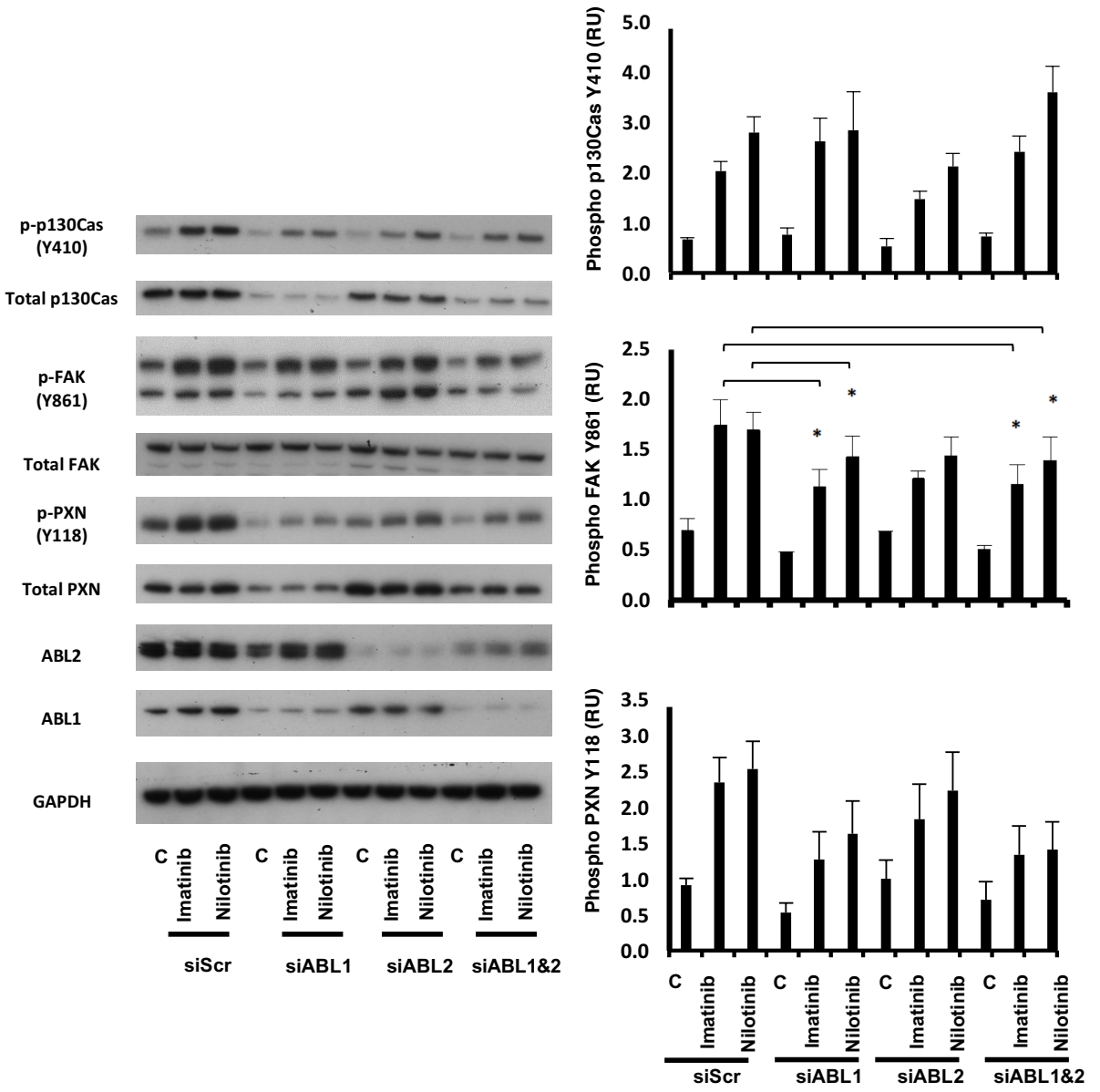


Figure 4.1.1. The effect of silencing ABL1 and ABL2 on observed imatinib- and nilotinib-induced tyrosine phosphorylation. U87MG cells were transfected with siRNA targeting ABL1 (siABL1), ABL2 (siABL2) or together at a concentration of 25 nM, or with 25 nM of a control scrambled siRNA (siScr). 48hr post transfection, cells were incubated in serum-free medium (SFM) for ~18hr prior to treatment with SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Densitometric analysis was followed by application of a Two-way ANOVA and the Student Newman Keuls post hoc test. Data from three independent experiments are presented as phosphorylation relative units (RU) (means +/- s.e.m.) normalised to total protein levels; *$p<0.05$ compared to imatinib and nilotinib siScr respectively.

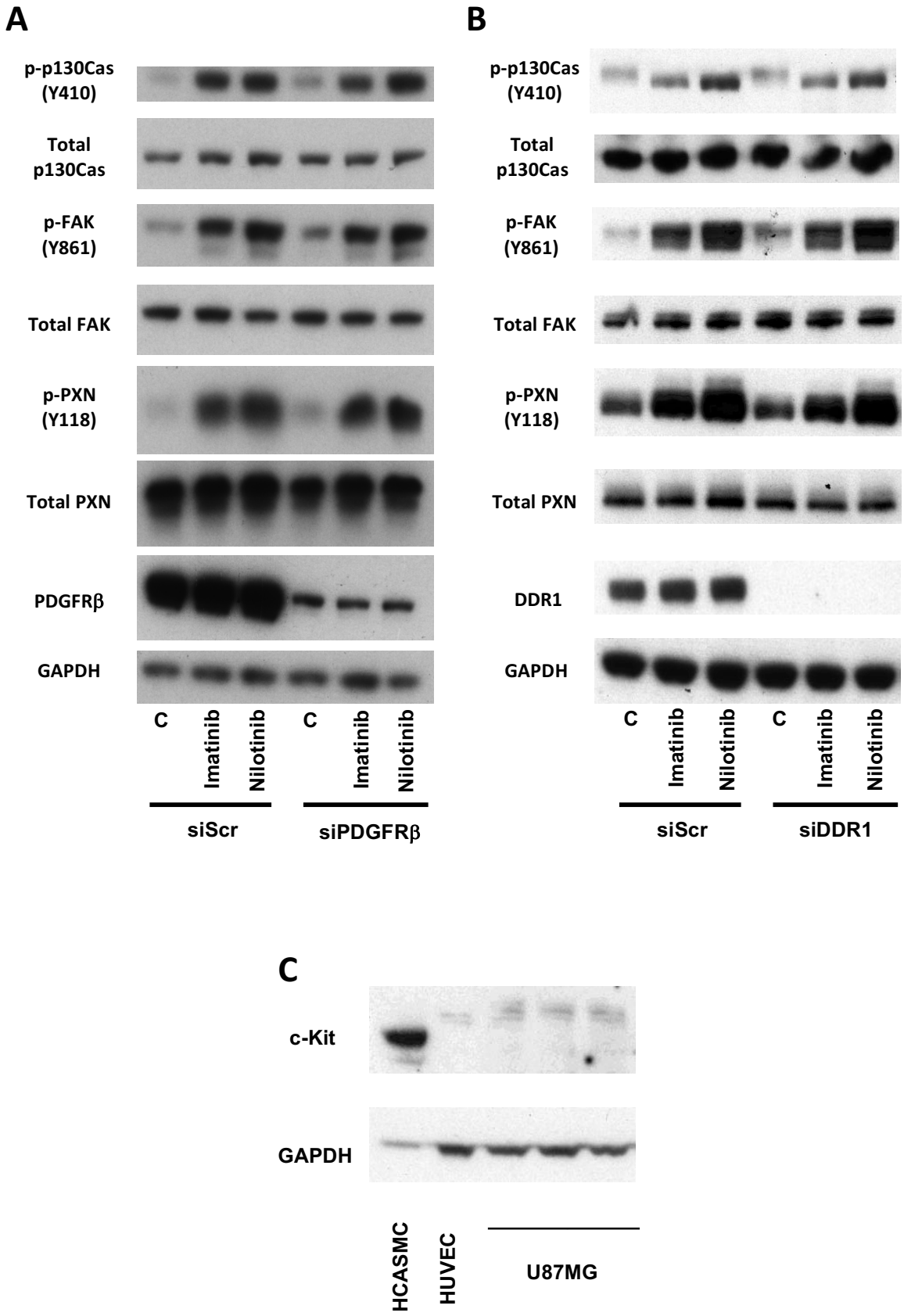

A
p-p130Cas (Y410)
Total p130Cas
p-FAK (Y861)
Total FAK
p-PXN (Y118)
Total PXN
PDGFRβ
GAPDH
C
Imatinib
Nilotinib
C
Imatinib
Nilotinib
siScr
siPDGFRβ
B
p-p130Cas (Y410)
Total p130Cas
p-FAK (Y861)
Total FAK
p-PXN (Y118)
Total PXN
DDR1
GAPDH
C
Imatinib
Nilotinib
C
Imatinib
Nilotinib
siScr
siDDR1
C
c-Kit
GAPDH
HCASMC
HUVEC
U87MG

Figure 4.1.2 PDGFRβ, DDR1 and c-Kit are not required for imatinib and nilotinib stimulated increases in p130Cas, FAK and PXN tyrosine phosphorylation. U87MG cells were transfected with siRNA targeting (**A**) PDGFR Beta (siPDGFRβ) or (**B**) DDR1 (siDDR1). Cells were transfected at a concentration of 25 nM siRNA, or with 25 nM of a control scrambled siRNA (siScr). 48hr post transfection, cells were incubated in SFM for ~18hr prior to treatment with SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. (**C**) U87MG cells, human umbilical vein endothelial cells (HUVECs) and human coronary artery smooth muscle cells (HCASMCs) were blotted for c-Kit and GAPDH (as a loading control). Only the smooth muscle cells expressed detectable levels of c-Kit.

4.2 Increased p130Cas, FAK and PXN tyrosine phosphorylation is dependent on p130Cas expression and FAK kinase activity

By contrast to the findings that drug-induced increases in tyrosine phosphorylation are not dependent on known targets, observed increases in phosphorylation are dependent on p130Cas expression. Targeted knockdown of p130Cas with two different siRNAs significantly reduced tyrosine phosphorylation of FAK and PXN induced by imatinib and nilotinib (**Fig. 4.2.1**). Furthermore, drug-induced 3D invasion of U87MG cells is also dependent on p130Cas expression. Spheroids were generated using cells treated with siRNA to p130Cas, which significantly reduced radial invasion induced by imatinib and nilotinib (**Fig. 4.2.2**).

To assess whether FAK is required for observed increases in tyrosine phosphorylation U87MG cells were treated with PF-573228 (PF-228), a potent inhibitor of FAK. PF-228 inhibits FAK phosphorylation on tyrosine residue Y397 with an IC_{50} of 30–100 nM and displays a 50-250-fold selectivity for FAK over other kinases (Slack-Davis et al., 2007). Treatment of U87MG cells with the FAK inhibitor PF-228at 5 μM significantly reduced the imatinib and nilotinib stimulated increases in p130Cas and PXN tyrosine phosphorylation (**Fig. 4.2.3**). Treatment with PF-228 also significantly reduced 3D radial invasion induced by imatinib and nilotinib (**Fig. 4.2.4**).

Because p130Cas and FAK are reported to exist in multi-protein complexes that are required for cell motility (Barrett et al., 2013), their association in glioma cells was examined. Immunoprecipitation of p130Cas and subsequent immunoblotting showed that FAK and PXN are both constitutively complexed with p130Cas in U87MG cells. These complexes are not affected by imatinib or nilotinib treatment (**Fig. 4.2.5**).

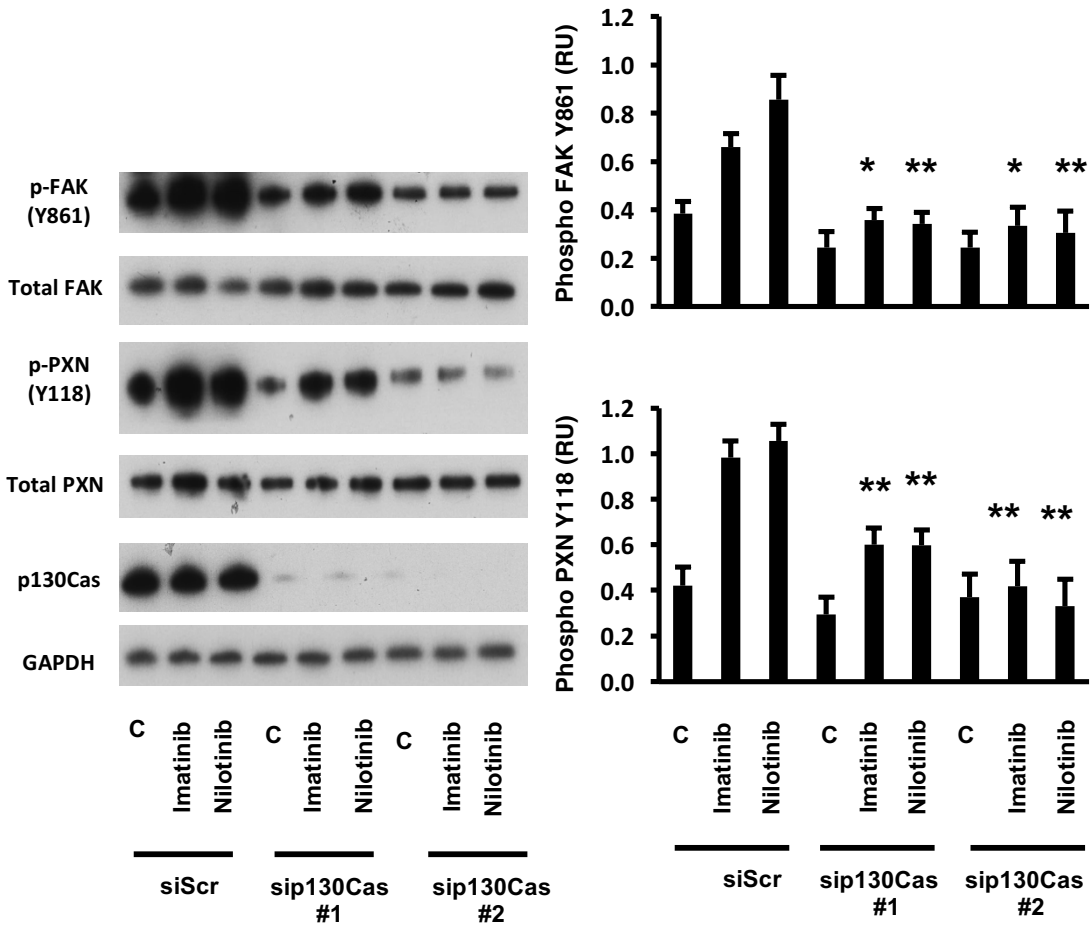


Figure 4.2.1 Increased p130Cas, FAK and PXN tyrosine phosphorylation is dependent on p130Cas expression. U87MG cells were transfected with siRNA targeting p130Cas (sip130Cas) at a concentration of 25 nM, or with 25 nM of a control scrambled siRNA (siScr). 48hr post transfection, cells were incubated in SFM for ~18hr prior to treatment with SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Data from three independent experiments are presented as phosphorylation relative units (RU) (means +/- s.e.m.) normalised to total protein levels; *p<0.05, **p<0.01 compared to imatinib and nilotinib siScr respectively.

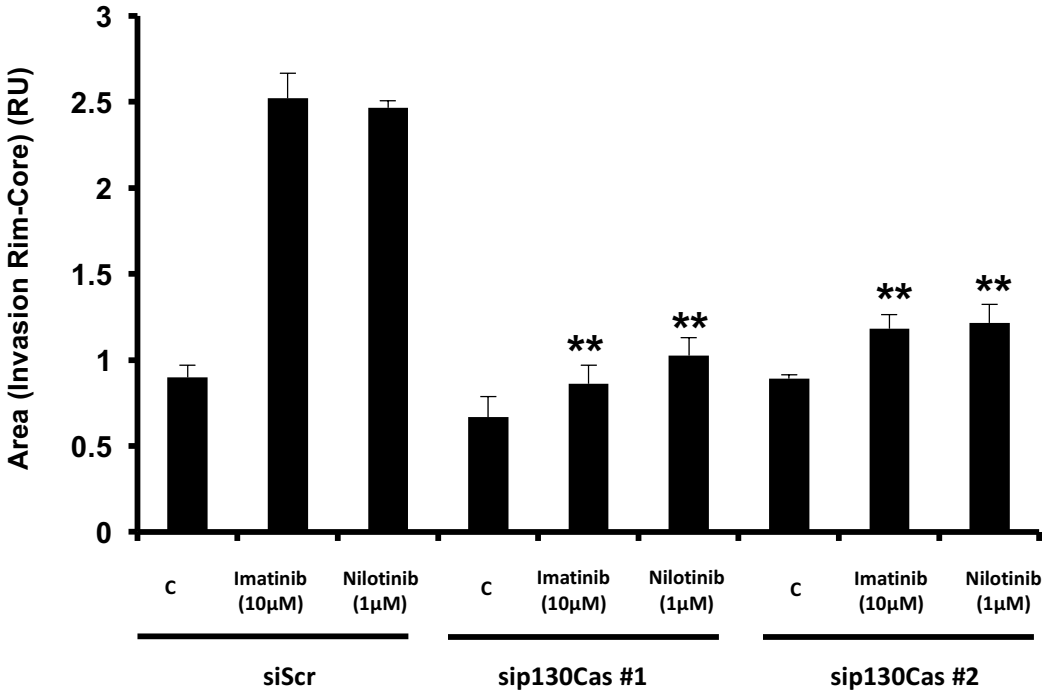


Figure 4.2.2 Imatinib- and nilotinib-induced radial invasion is dependent on the expression of p130Cas. Spheroids were generated using U87MG cells treated with siRNA to p130Cas (sip130Cas) or control scrambled siRNA (siScr). 24 hours after spheroid production, spheroids were imbedded in a collagen gel and incubated in either SFM & DMSO vehicle control (C) or 10 μM imatinib or 1 μM nilotinib for an additional 48 hours. Spheroids were fixed in 4% PFA and invasion was determined by measuring the area corresponding to the invasion rim minus the area of the core for at least 3 different spheroids per condition. Data from three independent experiments are presented as relative area units (RU) (means +/- s.e.m.); **$p < 0.01$ compared to imatinib and nilotinib siScr respectively.

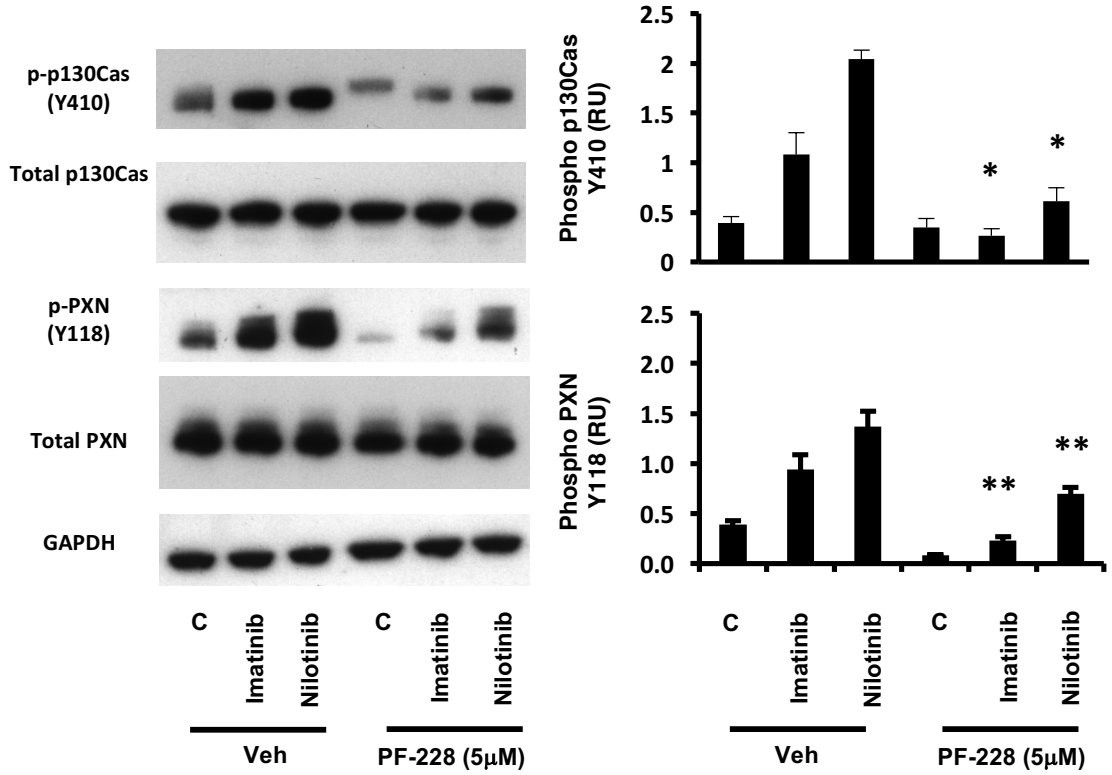


Figure 4.2.3 Increased p130Cas and PXN tyrosine phosphorylation is sensitive to treatment with a FAK kinase inhibitor. U87MG cells (~80% confl uent) were incubated in SFM for ~18hr prior to pre-incubation for 30 min with 5 μM PF-228 or 0.05% DMSO vehicle (Veh) prior to treatment with SFM & DMSO control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Data from three independent experiments are presented as phosphorylation relative units (RU) (means +/- s.e.m.) normalised to total protein levels; *p<0.05, **p<0.01 compared to imatinib and nilotinib DMSO vehicle respectively.

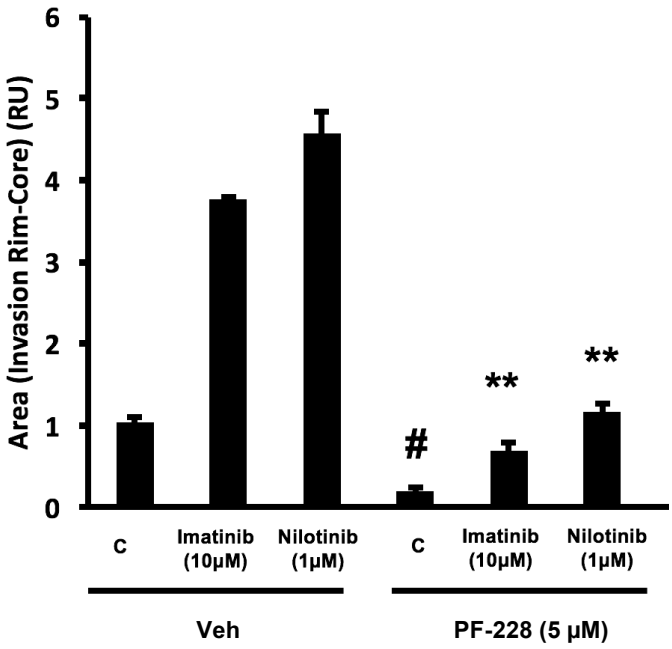


Figure 4.2.4 Imatinib- and nilotinib-induced increases in radial invasion are sensitive to treatment with a FAK kinase inhibitor. Spheroids derived from U87MG cells were imbedded in collagen containing either SFM & DMSO vehicle control (Veh) or 5 μM PF-228 with either DMSO control (C), 10 μM imatinib, or 1 μM nilotinib. After 48 hours spheroids were fixed in 4% PFA and invasion was determined by measuring the area corresponding to the invasion rim minus the area of the core for at least 3 different spheroids per condition. Data from three independent experiments are presented as relative area units (RU) (means +/− s.e.m.). **$p < 0.01$ compared to imatinib and nilotinib DMSO vehicle control respectively. #$p>0.05$ compared to control DMSO vehicle.

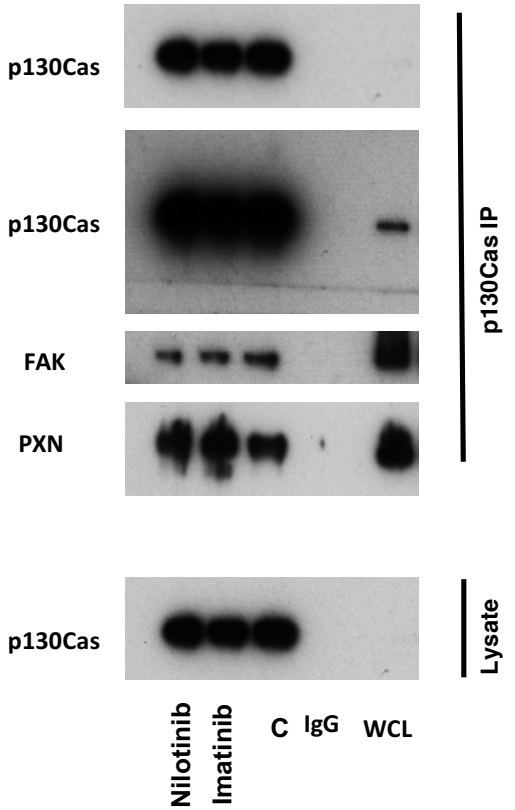


Figure 4.2.5 Co-immunoprecipitation of p130Cas, FAK, and PXN in U87MG cells. U87MG cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with either SFM & DMSO vehicle control (C), 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then immunoprecipitated with anti-p130Cas antibody. Purified complexes and corresponding total cell lysates were separated by SDS-PAGE, and probed with the indicated antibodies. IgG control and a whole cell lysate (WCL) were also included.

4.3 Increased tyrosine phosphorylation is not affected by modulation of the RAS / RAF / MER / ERK signalling pathway

Following the finding that known targets of imatinib and nilotinib are not required for drug-induced increases in tyrosine phosphorylation, the potential involvement of other key components of the pathways mediating motility was investigated.

It has recently been reported that imatinib and nilotinib treatment leads to the activation of MEK and ERK in colorectal, pancreatic, lung and melanoma cancer cell lines at physiologically relevant concentrations (Packer et al., 2011). ERK signalling is also implicated in meditating signalling pathways required for migration of glioma cells (Lind et al., 2006, Zohrabian et al., 2009). The effect of imatinib and nilotinib treatment on ERK activation in U87MG cells was examined. Although imatinib treatment increased levels of phosphorylated ERK, nilotinib treatment had no effect (**Fig. 4.3.1A**). U0126 is a selective MEK-1 and MEK-2 direct inhibitor (Favata et al., 1998). Even though U0126 completely abolished levels of phosphorylated ERK in all samples treated, it had no effect on imatinib and nilotinib stimulation of p130Cas, FAK, and PXN tyrosine phosphorylation (**Fig. 4.3.1A**). U0126 treatment had no significant effect on radial invasion in imatinib and nilotinib treated spheroids, although U0126 alone resulted in a small but significant increase in radial invasion compared with SFM vehicle control (**Fig. 4.3.2**).

RAS is a small GTPase, which causes downstream activation of tyrosine kinases RAF, MEK, and ERK. *RAS* genes (*HRAS, KRAS, NRAS*) are mutated in about 30% of human cancers. It has been reported that imatinib and nilotinib can bind to BRAF and CRAF leading to the formation of RAF hetero- and homo-dimers, and stimulate paradoxical activation of BRAF and CRAF in the presence of activated RAS (Packer et al., 2011). The possibility of a RAF-dependent, MEK/ERK-independent pathway leading to increased tyrosine phosphorylation of p130Cas, FAK and PXN, was therefore considered. However, silencing of BRAF or CRAF either alone or together had no effect on imatinib and nilotinib stimulated increases in tyrosine phosphorylation (**Fig. 4.3.1B**).

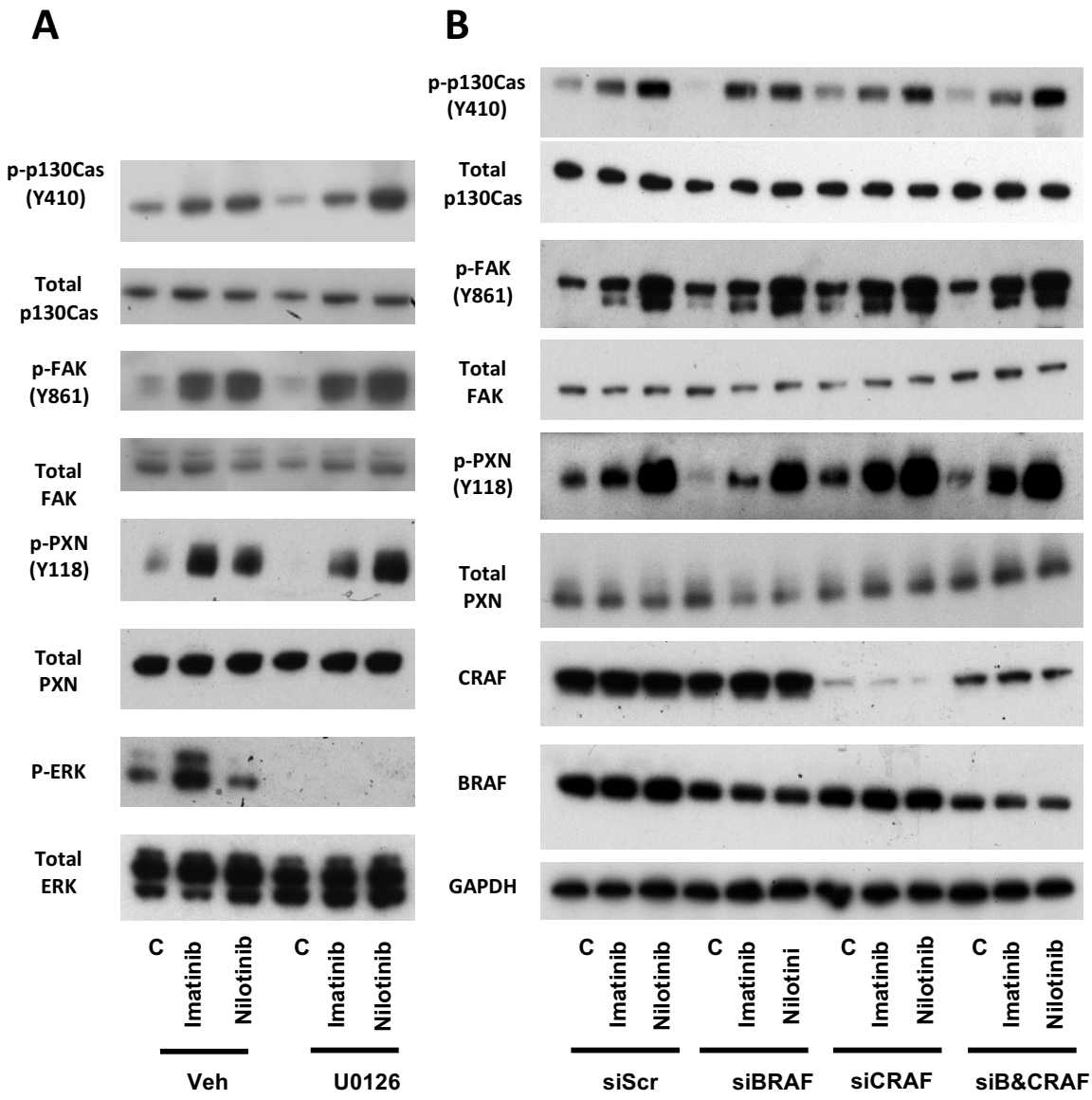


Figure 4.3.1 Pharmacological inhibition of ERK or treatment with BRAF and CRAF siRNA does not affect imatinib- and nilotinib-induced increases in p130Cas, FAK and PXN tyrosine phosphorylation. (**A**) U87MG cells (~80% confluent) were incubated in SFM for ~18hr prior to pre-incubation for 30 min with 10 μM U0126 or DMSO vehicle (Veh) prior to treatment with SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. (**B**) U87MG cells were transfected with siRNA targeting BRAF (siBRAF), CRAF (siCRAF) or together at a concentration of 25 nM, or with 25 nM of a control scrambled siRNA (siScr). 48hr post transfection, cells were incubated in SFM for ~18hr prior to treatment with SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies.

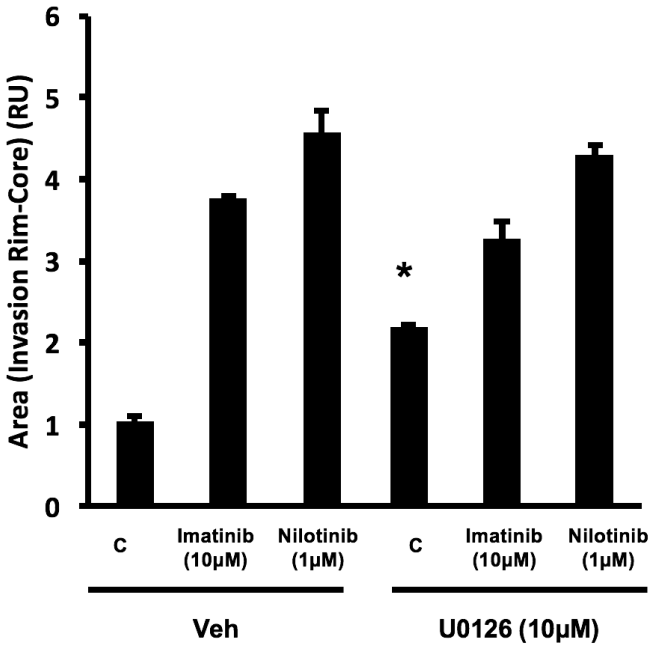


Figure 4.3.2 Imatinib- and nilotinib-induced increases in radial invasion are not affected by treatment with a MEK-1 and MEK-2 inhibitor. Spheroids derived from U87MG cells were imbedded in collagen containing either SFM & DMSO vehicle control (Veh) or 10 μM U0126, with either DMSO control (C), 10 μM imatinib, or 1 μM nilotinib. After 48 hours spheroids were fixed in 4% PFA and invasion was determined by measuring the area corresponding to the invasion rim minus the area of the core for at least 3 different spheroids per condition. Data from three independent experiments are presented as relative area units (RU) (means +/− s.e.m.). *$p < 0.05$ compared to control DMSO vehicle.

4.4 Src activity does not mediate increases in tyrosine phosphorylation in response to treatment with imatinib or nilotinib

Src directly phosphorylates p130Cas, FAK and PXN in response to growth factor stimulation, and provides a link between multiple signalling pathways that regulate invasion (Barrett et al., 2013). Imatinib has also been shown to bind Src, albeit at a much lower affinity, despite the fact that residues interacting with imatinib in crystal structures of ABL are conserved in Src (Deininger et al., 2005, Seeliger et al., 2007). The role of Src in mediating imatinib- and nilotinib-induced increases in tyrosine phosphorylation was investigated using PP2, a potent tyrosine kinase inhibitor selective for the Src family of tyrosine kinases (Hanke et al., 1996). U87MG cells were pre-treated with PP2 prior to treatment with imatinib or nilotinib. PP2 treatment leads to a complete abrogation of both basal and drug induced tyrosine phosphorylation of p130Cas, FAK and PXN (**Fig. 4.4.1**). This finding is not surprising given that Src exists at focal adhesions complexed with FAK and p130Cas, and that Src phosphorylates p130Cas and FAK (Sharma and Mayer, 2008). Because Src is required for p130Cas phosphorylation and Src phosphorylation at Y861 site enhances FAK binding to p130Cas, Src inhibition will intervene with basal levels of tyrosine phosphorylation of FAK and p130Cas. An alternative approach for assessing whether Src may be mediating observed drug-induced effects is to examine the effect of drug stimulation on levels of phosphorylation of the Src activating tyrosine residue Y416. Stimulation of U87MG cells with imatinib or nilotinib did not result in changes in the levels of Y416 phosphorylation, suggesting that imatinib and nilotinib are not affecting the activation of Src (**Fig. 4.4.2**).

The overall sequences of Src family kinases (SFKs), with the exception of the N-terminal ~50 residues, is highly conserved among the nine family members. Full activation of SFKs requires dephosphorylation at the exposed tyrosine Y527 residue, which stabilises the active conformation. Subsequent autophosphorylation at tyrosine Y416 locks the catalytic pocket in its active conformation. The activated SFK can then phosphorylate substrates, such as FAK and p130Cas. When the signalling response is terminated the SFKs are inactivated by phosphorylation at tyrosine residue Y527 (Okada, 2012). The NRTK CSK catalyses the

phosphorylation of the regulatory C-terminal tail tyrosine of Src, the tyrosine residue Y527, to inactivate Src. The negative regulation of Src is critical to cell survival and CSK is expressed in all mammalian cells (Roskoski, 2005). When Y527 is phosphorylated by CSK, Src adopts the inactive conformation which is stabilized by two intramolecular interactions that affect the configuration of the catalytic pocket: Y527 binds its own SH2 domain, and the SH2 domain binds to the SH3 domain (Okada, 2012). In addition to SFKs, several signalling proteins are reported to interact with CSK. These include FAK and PXN. In fibroblasts, PXN and FAK colocalise at focal adhesions and bind the CSK SH2 domain (Sabe et al., 1994).

If imatinib and nilotinib were directly targeting CSK, this could provide a mechanism for increased Src activation, which would result in increased tyrosine phosphorylation of p130Cas, FAK and PXN. However, preliminary findings suggest that silencing CSK with siRNA does not affect drug-induced tyrosine phosphorylation of p130Cas, FAK and PXN (**Fig.4.4.3**).

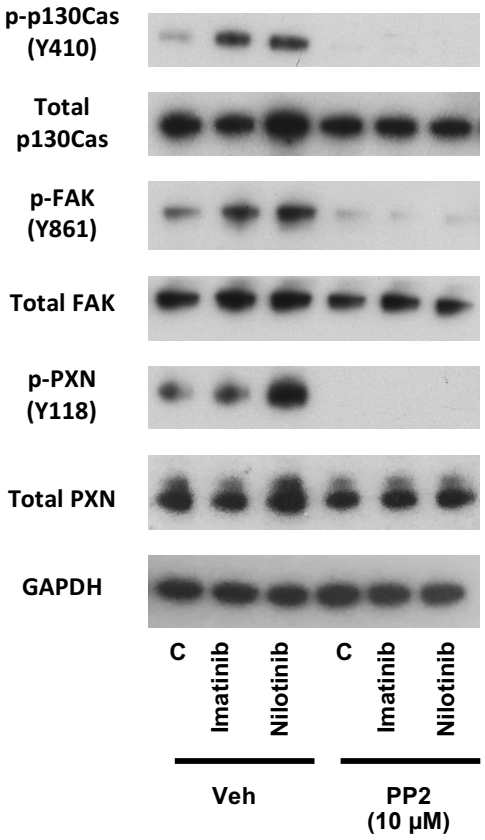


Figure 4.4.1 p130Cas, FAK and PXN tyrosine phosphorylation are sensitive to treatment with a Src kinase inhibitor. U87MG cells (~80% confluent) were incubated in SFM for ~18hr prior to pre-incubation for 20 min with 10 μM PP2 or DMSO vehicle (Veh) prior to treatment with SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies.

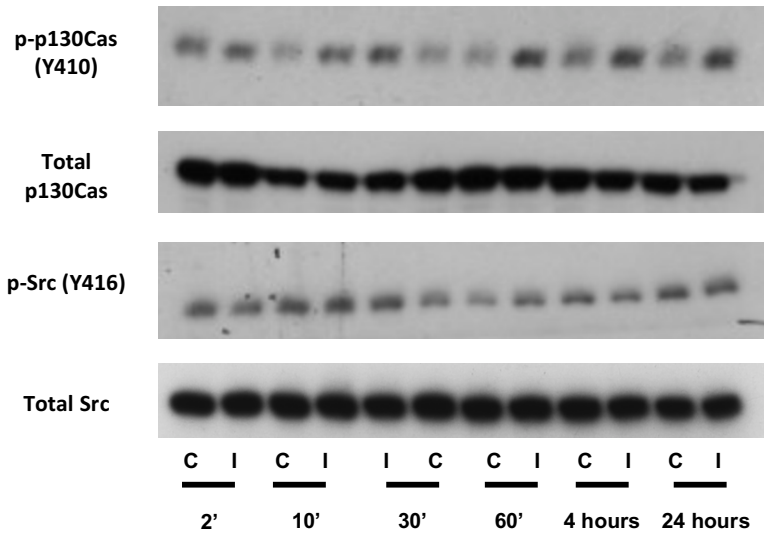


Figure 4.4.2 Imatinib and nilotinib treatment does not affect tyrosine phosphorylation of Src at residue Y416. U87MG cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with either SFM & DMSO vehicle control (C), or 10 μM imatinib (I) for the specified amount of time. Cell lysates were then prepared, blotted, and probed with the indicated antibodies.

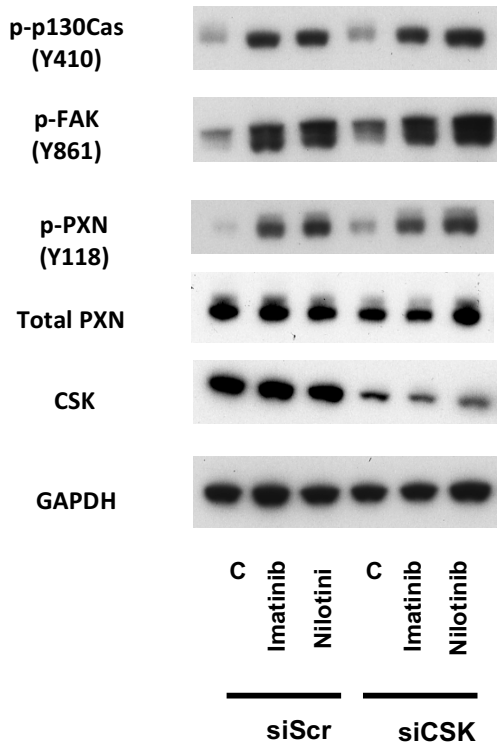


Figure 4.4.3 Knockdown of CSK does not affect imatinib and nilotinib stimulated increases in tyrosine phosphorylation. U87MG cells were transfected with siRNA targeting CSK (siCSK) at a concentration of 25 nM, or with 25 nM of a control scrambled siRNA (siScr). 48hr post transfection, cells were incubated in SFM for ~18hr prior to treatment with SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Blots are representative of *n*=2 experiments.

4.5 Increased tyrosine phosphorylation is not affected by silencing integrins β1 and β3 and NCK

Integrins are known to mediate p130Cas and FAK tyrosine phosphorylation through beta 1 and beta 3 subunits. Integrin receptor activation leads to FAK autophosphorylation at tyrosine residue Y397, which recruits Src and p130Cas (Sharma and Mayer, 2008, Barrett et al., 2013). The possible involvement of integrin signalling in mediating imatinib and nilotinib stimulated tyrosine phosphorylation was investigated. U87MG cells were treated with two different siRNAs to either ITGB1 or ITGB3 integrins. Silencing resulted in a marked reduction in integrin protein expression, but had no effect on drug-induced tyrosine phosphorylation of p130, FAK and PXN (**Figure 4.5.1**). Knockdown of β1 and β3 integrins in this experiment did not positively confirm involvement of β1 and β3 integrins in mediating the effects of imatinib and nilotinib on tyrosine phosphorylation.

NCK (non-catalytic region of tyrosine kinase adaptor protein) is a ubiquitously expressed cytosolic adaptor protein made up of one SH2 domain and three SH3 domains. It plays an important role in mediating motility by linking RTKs and NRTKs to actin cytoskeleton reorganising proteins. The SH2 domain associates with phosphotyrosine residues of a number of proteins, including BCR-ABL, EGFR and PDGFR. The SH3 domain recruits proline-rich proteins to the plasma membrane (Buday et al., 2002). The possible involvement of NCK in mediating drug-induced effects was investigated. However, preliminary findings suggest that silencing NCK does not affect imatinib- and nilotinib-induced increases in tyrosine phosphorylation (**Appendix Fig. A3**).

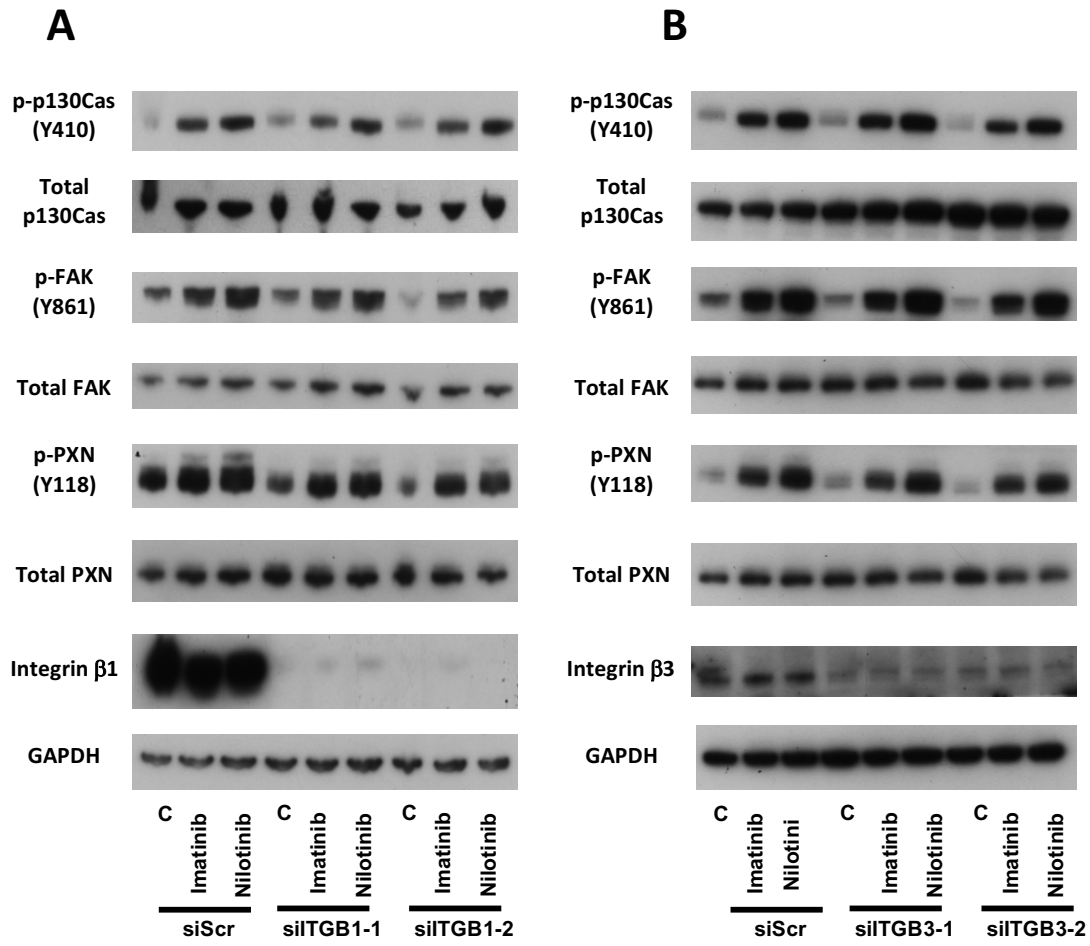


Figure 4.5.1 Treatment with β1 or β3 siRNA does not affect drug-induced tyrosine phosphorylation of p130Cas, FAK and PXN. (**A**) U87MG cells were transfected with two siRNA targeting integrin β1 (siITGB1) at a concentration of 25 nM, or with 25 nM of a control scrambled siRNA (siScr). (**B**) U87MG cells were transfected with two siRNA targeting integrin β3 (siITGB3) at a concentration of 25 nM, or with 25 nM of a control scrambled siRNA (siScr). (**A-B**) 48hr post transfection, cells were incubated in serum-free medium (SFM) for ~18hr prior to treatment with SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies.

4.6 Discussion

The major known targets of imatinib and nilotinib are ABL, ABL2 (ARG), DDR1, PDGFR and c-Kit (Hantschel et al., 2008). The effect of silencing these targets on imatinib- and nilotinib-induced tyrosine phosphorylation was evaluated. Preliminary data suggests that the effects of imatinib and nilotinib on p130Cas, FAK and PXN tyrosine phosphorylation are unaffected by treatment with siRNA targeting ABL1, ABL2, DDR1 and PDGFRβ.

Knockdown of ABL1, ABL2 or both ABL1 and ABL2 led to a decrease in total levels of p130Cas and PXN, making it difficult to evaluate the role of ABL1 and ABL2 in mediating drug-induced tyrosine phosphorylation. Nevertheless, the data revealed that there is no significant effect of knockdown on imatinib and nilotinib induced tyrosine phosphorylation of p130Cas and PXN. The levels of phospho-p130Cas in the ABL1, ABL2 or both ABL1 and ABL2 siRNA-treated cells did not significantly differ from the siScr-treated cells. Silencing of ABL1 or both ABL1 and ABL2 did however lead to a significant reduction in phosphorylation of FAK. Interestingly no decrease in basal phosphorylation of FAK in the control treated cells was detected. It is proposed that the reduced p130Cas expression in cells treated with siRNA is sufficient to facilitate drug induced FAK phosphorylation. This is supported by the findings that varying levels of p130Case knockdown efficiency between two different siRNAs causes differences in the reduction of imatinib and nilotinib induced tyrosine phosphorylation of FAK and PXN (Fig. 4.2.1). Immunoprecipitation of p130Cas revealed the interdependence of p130Cas, FAK and PXN that constitutively complex together in U87MG cells. Importantly, imatinib and nilotinib did not affect this interdependence as drug stimulation did not have an effect on complexes.

There is evidence that PDGF autocrine loops contribute to the transformed phenotype of human astrocytomas. PDGF A and B ligand genes are expressed in almost all glioma cell lines and in isolates of human malignant astrocytoma (Kilic et al., 2000). Dominant-negative mutants of the PDGF ligands A and B have been found to inhibit growth of U87MG cells (Shamah et al., 1993). Imatinib was considered a rational drug candidate for the treatment of the GBM based on the evidence that PDGFR is implicated in driving pathology (Raymond et al., 2008). Imatinib has previously been found to

inhibit PDGFR tyrosine phosphorylation in tumours generated by injecting U87MG cells into mice. Oral administration of imatinib was alsofound to reduce tumour growth in this mouse model of GBM (Kilic et al., 2000). Interestingly, findings here show that knockdown of PDGFRβ does not affect the imatinib- and nilotinib-induced tyrosine phosphorylation of p130Cas, FAK and PXN in U87MG cells. U87MG cells mainly express PDGFRβ, and the expression of PDGFRa is very weak (Gross et al., 2006). Findings suggest that PDGFR does not mediate imatinib- and nilotinib-induced tyrosine phosphorylation in GBM cells. Imatinib has failed clinical trials for glioblastoma, where it shows no significant antitumour activity or extension of survival (Wen et al., 2006, Raymond et al., 2008). Findings here indicate unforeseen effects of imatinib and nilotinib on cell motility that may be independent of PDGFR, which could contribute to the lack of clinical efficacy observed in these trials.

Knockdown of DDR1, another major target of imatinib and nilotinib (Hantschel et al., 2008), did not affect drug-induced tyrosine phosphorylation either. The lack of expression of c-Kit in U87MG cells indicates that c-Kit is not mediating drug-induced tyrosine phosphorylation either. These findings reveal that alternative pathway(s) are being activated by the drugs that are not dependent on known targets.

The possibility that imatinib and nilotinib signal via the RAS/RAF/MEK/ERK pathway to stimulate phosphorylation was investigated. Imatinib leads to increased ERK phosphorylation in U87MG cells, whereas nilotinib does not. This suggests that imatinib and nilotinib may be activating different signalling pathways in these cells. In healthy cells, the ERK pathway regulates survival, proliferation and differentiation. In cancer, ERK signalling downstream of Ras is associated with invasive growth and metastasis, whereby deregulated and constitutive activation promotes excessive proliferation and survival (Heidorn et al., 2010, Viala and Pouyssegur, 2004). Pharmacological inhibition of MEK with U0126 had no effect on imatinib- and nilotinib-stimulated tyrosine phosphorylation or migration. Treatment with BRAF or CRAF siRNA, either alone or together, did not affect increased tyrosine phosphorylation in response to drug treatment either. To confirm that knockdown of BRAF and CRAF was sufficient to block downstream signalling, lysates should be probed for phospho-ERK. However, preliminary results suggest that the RAS/RAF/MEK/ERK pathway does not mediate imatinib- or nilotinib-induced effects on tyrosine phosphorylation and migration in U87MG cells.

Imatinib and nilotinib have been reported to activate MEK and ERK in colorectal cancer, pancreatic cancer, lung cancer and melanoma cell lines at concentrations as low as 100nM. The study also revealed that in cells derived from patients with CML expressing the BCR-ABL T315I gatekeeper mutation, imatinib and nilotinib induced BRAF binding to CRAF and induced activation of MEK and ERK (Packer et al., 2011). By contrast, findings here suggest that imatinib and nilotinib exert effects on the p130Cas-FAK pathway distinct from the MEK pathway, which is not required for drug-induced tyrosine phosphorylation or migration.

The NRTK Src is the main kinase that phosphorylates p130Cas, FAK and PXN (Barrett et al., 2013). This is supported by findings in this study that inhibition of Src with PP2 abolished basal levels of p130Cas, FAK and PXN tyrosine phosphorylation. Imatinib and nilotinib treatment did not result in any changes in the levels of tyrosine phosphorylation of the activating Src residue tyrosine Y416, suggesting that Src activity is not affected by drug treatment. A possible mechanism of imatinib- and nilotinib-induced tyrosine phosphorylation of p130Cas, FAK and PXN, is the direct inactivation of an inhibitory kinase, which under physiological conditions inhibits a downstream kinase responsible for phosphorylation of the three proteins. When the Src inhibitory residue Y527 is phosphorylated by CSK, Src adopts the inactive conformation (Okada, 2012). If imatinib and nilotinib inhibit CSK, this would prevent the inhibition of Src, and could lead to increased downstream tyrosine phosphorylation of p130Cas, FAK and PXN. However, preliminary results suggest that silencing CSK does not have an effect on drug-induced phosphorylation. Findings suggest that although Src is required for basal phosphorylation of p130Cas, FAK, and PXN, it does not mediate imatinib- and nilotinib-driven phosphorylation.

FAK inhibition with PF-228 led to a significant decrease in the basal level of radial invasion, supporting the role of FAK in mediating migration. Results show that imatinib- and nilotinib-induced tyrosine phosphorylation of p130Cas and PXN is sensitive to the inhibition of FAK kinase activity. Further experiments looking at the effect of treatment with additional pharmacological inhibitors and siRNA targeting FAK could be performed to confirm the role of FAK in mediating drug-induced phosphorylation.

Preliminary findings suggest that pharmacological modulation of FAK may ameliorate the clinical management of GBM and the clinical management of CML with imatinib / nilotinib. Several small-molecule FAK TKIs are currently undergoing pre-clinical and clinical testing. TAE-226 is a pre-clinical compound that acts as a potent ATP-competitive inhibitor of FAK and inhibits FAK autophosphorylation at Y397. It has demonstrated strong anti-tumour activities in glioma, where it induces a concentration-dependent decrease in cellular proliferation (Lee et al., 2014a, Shi et al., 2007, Liu et al., 2007b).

Previous studies provide evidence of a role for FAK in promoting haematological malignancies. Aberrant expression of FAK and subsequent hyper-activation is detected in about half of acute myeloid leukaemias (Recher et al., 2004). FAK is constitutively activated in BCR-ABL-transformed cell lines and FAK silencing inhibits BCR–ABL-mediated leukaemic cell survival and proliferation (Gotoh et al., 1995, Le et al., 2009, Yin, 2011). The evidence that FAK silencing significantly reduces BCR-ABL-mediated cell survival *in vitro* provides compelling evidence that targeting FAK could provide therapeutic benefit. More recently it was found that TAE226 in combination with nilotinib provided greater growth inhibition than nilotinib alone *in vivo* in mice xenografted with Ph+ leukaemia cells (Hu and Slayton, 2014). Results here support previous findings that FAK inhibition in conjunction with imatinib or nilotinib treatment could ameliorate their clinical management.

5 The role of phosphatases in mediating drug effects

5.1 Investigating the role of protein tyrosine phosphatases

The phosphorylation of a particular residue can be affected by changing either the activity of a kinase, a phosphatase, or both (**Fig. 5.1.1**). One way in which imatinib and nilotinib treatment could lead to increased tyrosine phosphorylation could be via inhibition of an inhibitory tyrosine kinase, such as the example of CSK in relationship to Src as described in Chapter 4. However, drug treatment did not have an effect on Src activating residue Y416; and nor did knockdown of CSK have an effect on drug-induced tyrosine phosphorylation.

Another possible mechanism by which imatinib and nilotinib induce tyrosine phosphorylation of p130Cas, FAK and PXN and enhance migration, may involve indirect inactivation of a protein tyrosine phosphatase (PTP). It is possible that imatinib and nilotinib are targeting a kinase that, under physiological conditions, activates one or more PTP. By direct inhibition of a kinase, the TKIs may be preventing the downstream activation of a phosphatase. By causing downstream inhibition of phosphatase activity, imatinib and nilotinib would prevent dephosphorylation and thus drive upwards the level of phosphorylation (**Fig. 5.1.1**).

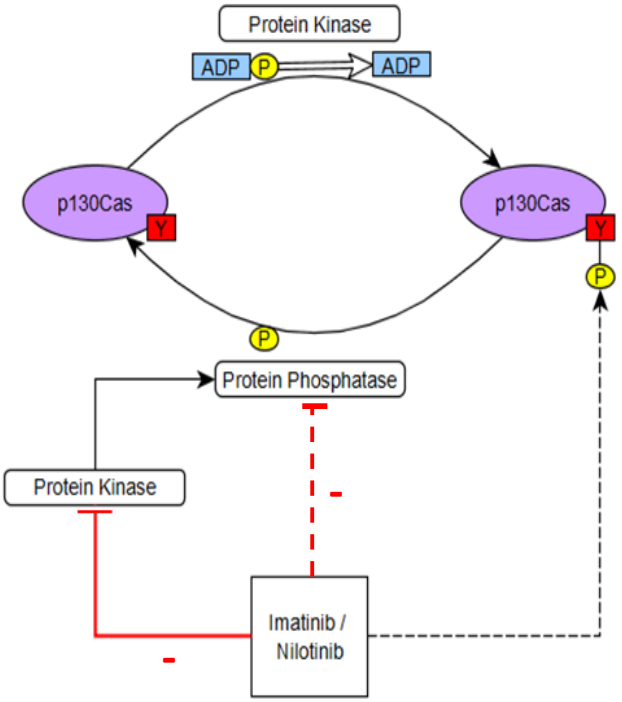


Figure 5.1.1 Possible mechanism of imatinib- and nilotinib-induced tyrosine phosphorylation. The net level of protein tyrosyl activity is balanced by the opposing actions of protein tyrosine kinases and protein tyrosine phosphatases. A protein kinase transfers phosphates from ATP to tyrosine residues on numerous substrates, including p130Cas. A protein phosphatase removes phosphate groups from the substrate, thus inactivating it and directly opposing the action of the kinase (Paul and Mukhopadhyay, 2004). Imatinib and nilotinib treatment leads to increased tyrosine phosphorylation of p130Cas (black dotted line). A possible mechanism by which imatinib and nilotinib exert effects is by the indirect inhibition a phosphatase (red dotted line); this could be achieved via inhibition of protein kinase (red solid line) that under physiological conditions activates a phosphatase (black solid line).

It is proposed that imatinib and nilotinib may be inducing their effects via indirect inhibition of a PTP. Dephosphorylation of p130Cas by PTP-PEST (encoded by PTPN12) is required for the disassembly of focal adhesions; SHP2 (encoded by PTPN11) is required for the activation of the Ras-ERK pathway in response to growth factors; PTP-1B (encoded by PTPN1) dephosphorylates the insulin receptor, EGFR and PDGFR (Ostman et al., 2006). The role of these PTPs in mediating drug-induced effects was investigated using siRNA.

If a particular phosphatase is being inhibited indirectly by imatinib / nilotinib, silencing the phosphatase should lead to similar increases in tyrosine phosphorylation that are observed upon drug treatment. Silencing PTP1B and SHP2 provided inconsistent data. Two different siRNAs against SHP2 led to varying levels of knockdown, and knockdown of SHP2 had different effects on p130Cas, FAK and PXN phosphorylation. SHP2 siRNA #6 led to an increase in tyrosine phosphorylation of FAK but not p130Cas. And although two different siRNAs against PTP1B both led to good knockdown, the two siRNA caused different effects on tyrosine phosphorylation: PTP1B siRNA #6 caused an increase in tyrosine phosphorylation of p130Cas, FAK and PXN, whereas siRNA #7 did not affect tyrosine phosphorylation (**Fig. 5.1.2A**). Silencing PTP-PEST with two different siRNAs led to a good knockdown. Silencing PTP-PEST did not cause a significant increase in basal levels of p130Cas or PXN phosphorylation; nor did silencing have an effect on imatinib- and nilotinib-induced increases in phosphorylation. This suggests that PTP-PEST is not involved in mediating the observed drug-induced effects on tyrosine phosphorylation (**Fig. 5.1.2B**).

The receptor protein tyrosine phosphatase α (PTPα; encoded by PTPRA) is implicated in integrin signalling, cell adhesion and proliferation. PTPα dephosphorylates Src at its inactivating residue Y527 and interacts with FAK (Ostman et al., 2006, Su et al., 1999). The potential involvement of PTPα was investigated by transfecting glioma cells with two different siRNAs. Knockdown efficiency was evaluated by immunoblotting for PTPRA tyrosine residue Y789, the residue whose phosphorylation has been shown to be required for targeting PTPα to focal adhesions (Lammers et al., 2000). Preliminary results show that although siRNA treatment led to good knockdown of PTPRA, it did not affect imatinib- and nilotinib-induced increases in tyrosine phosphorylation of p130Cas and PXN, suggesting that PTPα is not mediating drug-induced tyrosine phosphorylation (**Appendix Fig. A4**).

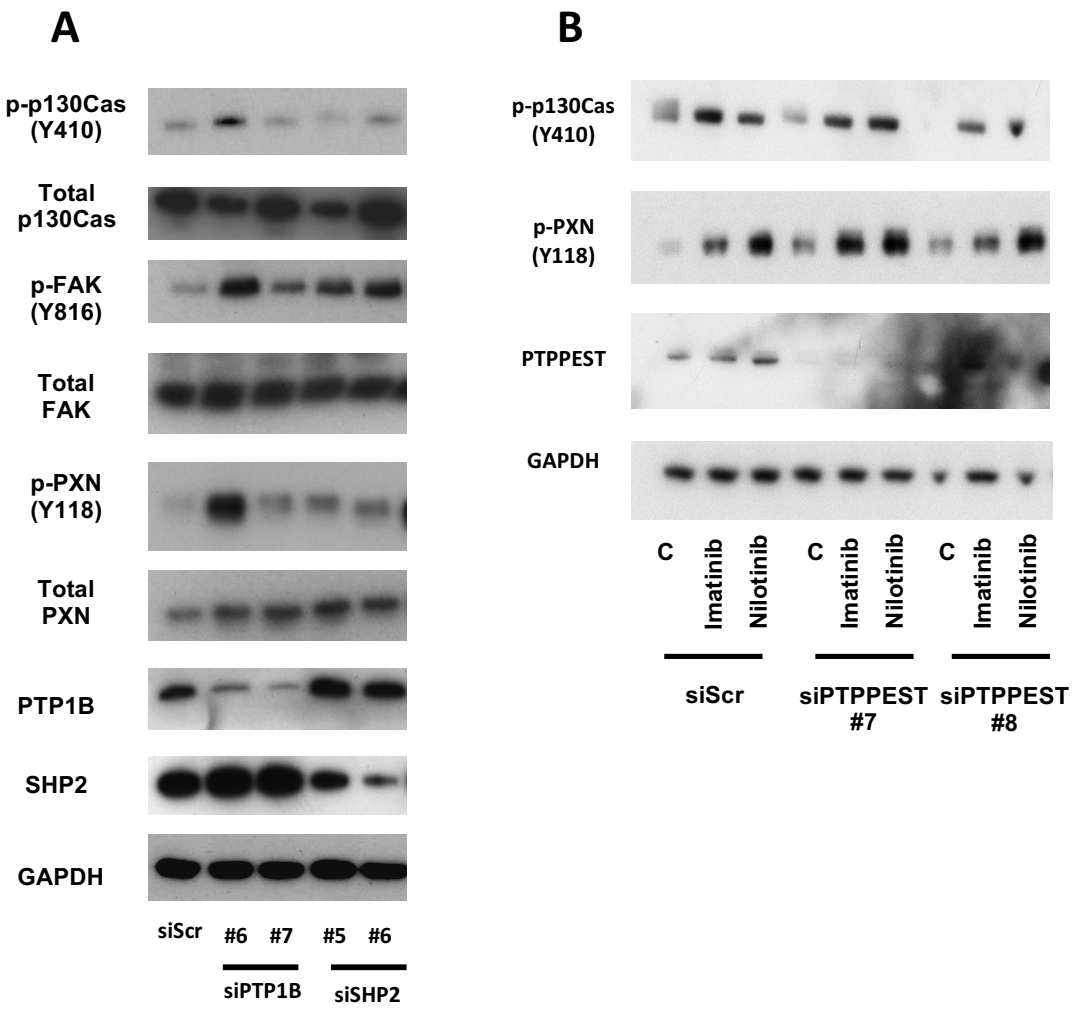


Figure 5.1.2 Effect of silencing PTP1B, SHP2 and PTP-PEST phosphatases on tyrosine phosphorylation. (**A**) U87MG cells were transfected with two different siRNAs targeting PTP1B (siPTP1B) and two different siRNAs targeting SHP2 (siSHP2) at a concentration of 25 nM, or with 25 nM of a control scrambled siRNA (siScr). 72hr post transfection, cell lysates were prepared, blotted, and probed with the indicated antibodies. (**B**) U87MG cells were transfected with two different siRNAs targeting PTP-PEST (siPTPPEST) at a concentration of 25 nM, or with 25 nM of a control scrambled siRNA (siScr). 48hr post transfection, cells were incubated in SFM for ~18hr prior to treatment with SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies.

5.2 The involvement of PP2A serine phosphatase in mediating imatinib and nilotinib stimulation of tyrosine phosphorylation

Protein phosphatase 2A (PP2A) is a ubiquitously expressed serine/threonine phosphatase which acts as a tumour suppressor and plays a crucial role in the regulation of cell cycle progression, survival, and differentiation. The loss of PP2A activity has been identified in some myeloid malignancies, including blast-phase CML and AML (Yang et al., 2012). It has been shown that PP2A is inactivated by long-term treatment (24h) with nilotinib in human hepatocellular carcinoma (HCC) cell lines (Yu et al., 2013). PP2A is known to interact and associate with p130Cas (Yokoyama and Miller, 2001b) and has been found to inhibit Src activity (Yokoyama and Miller, 2001a). The role of PP2A in imatinib- and nilotinib-induced tyrosine phosphorylation was examined using pharmacological modulators of PP2A activity. U87MG cells were treated with the potent PP2A activator FTY720 (Yang et al., 2012), and inhibitor okadaic acid (OA) (Garcia et al., 2002).

Pharmacological inhibition of PP2A with OA significantly increased the tyrosine phosphorylation of p130Cas, FAK and PXN in vehicle control cells. Conversely, pharmacological activation of PP2A using FTY720 strongly inhibited imatinib and nilotinib stimulated tyrosine phosphorylation of p130Cas, FAK and PXN (**Fig. 5.2.1**). p130Cas and PXN have been previously reported to associate with the catalytic subunit of PP2A (Ito et al., 2000, Yokoyama and Miller, 2001b). Co-immunoprecipitation of p130Cas and PP2A in U87MG cells revealed that PP2A associates with p130Cas. Association of PP2A and p130Cas is constitutive, and was unaffected by imatinib or nilotinib treatment compared to vehicle control (**Fig. 5.2.2**). The effect of modulating PP2A activity on imatinib- and nilotinib-induced 3D radial invasion was assessed. Treatment of U87MG spheroids with FTY720 caused a striking reduction in drug-induced 3D radial invasion (**Fig. 5.2.3**).

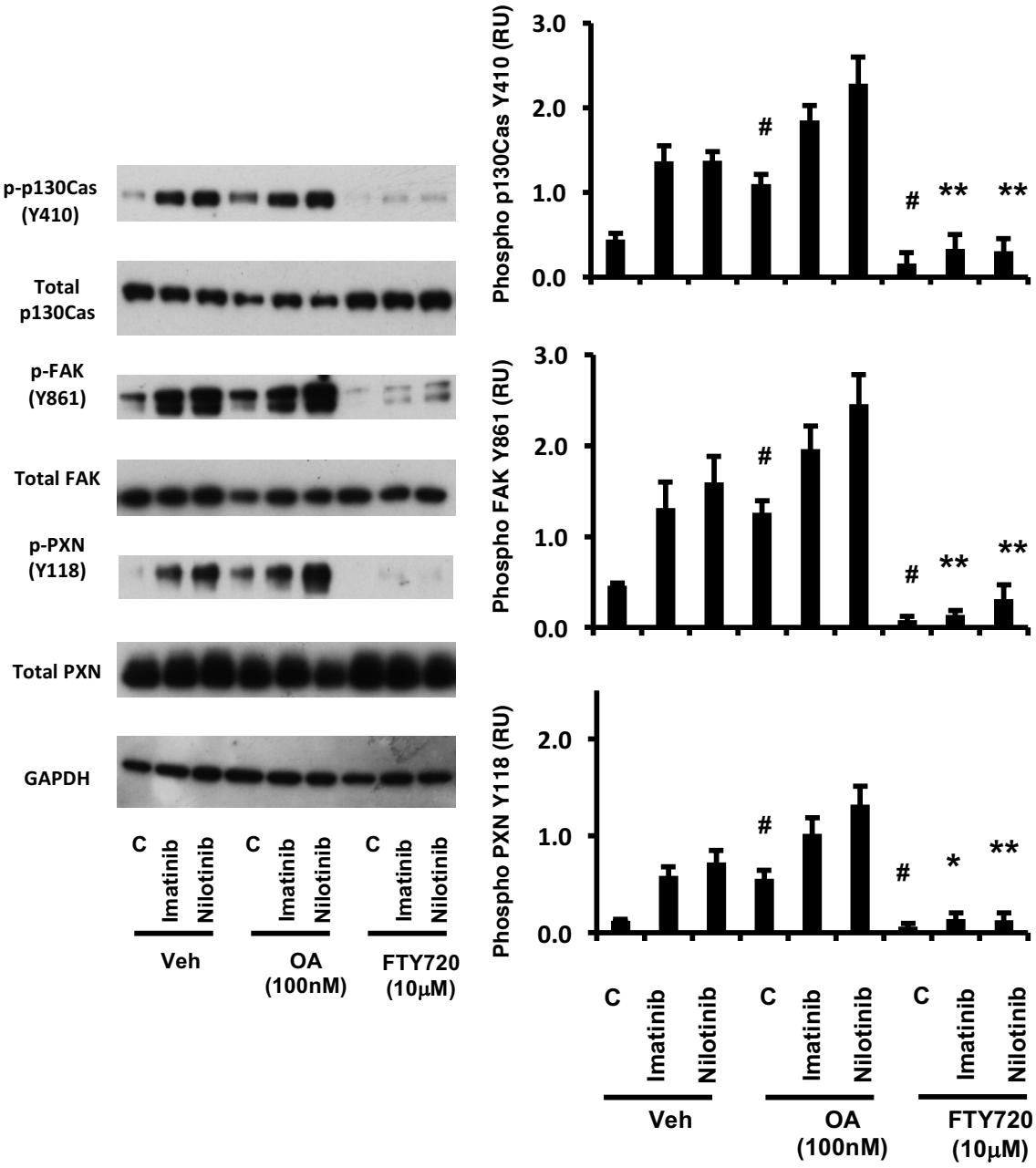


Figure 5.2.1 Drug-induced increases in tyrosine phosphorylation is mediated by PP2A activity. U87MG cells (~80% confluent) were incubated in SFM for ~18hr prior to pre-incubation for 30 min with 10 μM FTY720 or 100 nM okadaic acid (OA) or DMSO vehicle control (Veh) prior to treatment with SFM & DMSO control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Data from three independent experiments are presented as phosphorylation relative units (RU) (means +/- s.e.m.) normalised to total protein levels; *$p<0.05$, **$p<0.01$ compared to imatinib and nilotinib DMSO vehicle respectively. #$p>0.05$ compared to control DMSO vehicle.

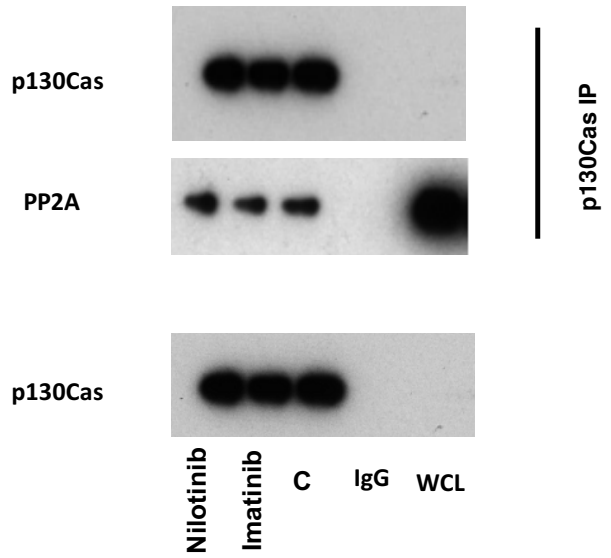


Figure 5.2.2 PP2A and p130Cas co-immunoprecipitation is not affected by imatinib and nilotinib treatment. U87MG cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with either SFM & DMSO vehicle control (C), 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then immunoprecipitated with anti-p130Cas antibody. Purified complexes were separated by SDS-PAGE, and probed with PP2A antibody. IgG control and a whole cell lysate (WCL) were also included.

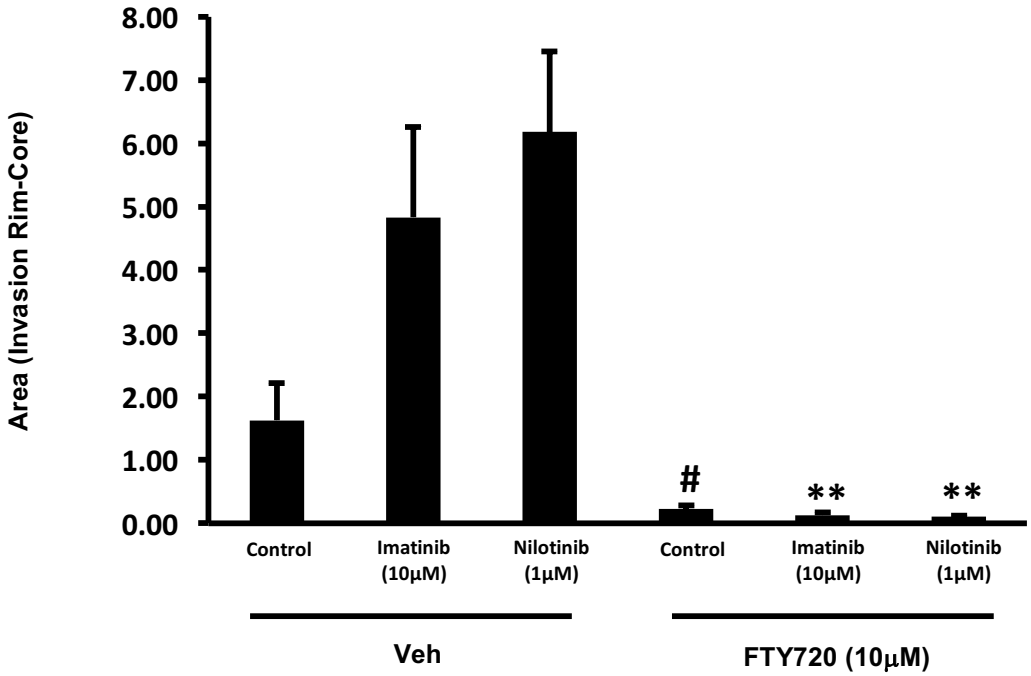


Figure 5.2.3 Drug-induced increases in three-dimensional radial invasion is regulated by PP2A activity. Spheroids derived from U87MG cells were imbedded in collagen containing either SFM & DMSO vehicle control (Veh) or 10 μM FTY720 with either DMSO control (C) or 10 μM imatinib or 1 μM nilotinib for 48 hours. Spheroids were fixed in 4% PFA and invasion was determined by measuring the area corresponding to the invasion rim minus the area of the core for at least 3 different spheroids per condition. Data from three independent experiments are presented as relative area units (RU) (means +/- s.e.m.). **$p < 0.01$ compared to imatinib and nilotinib DMSO vehicle control respectively. #$p>0.05$ compared to control DMSO vehicle.

5.3 Discussion

It is possible that imatinib and nilotinib inhibit one or more kinases, which under physiological conditions activate one or more phosphatase. In this context, drug-induced inhibition of the kinase would lead to a reduction in phosphatase activity, and subsequent reduction in dephosphorylation. The net result would be increased tyrosine phosphorylation (**Fig. 5.1.1**). Interestingly, findings implicate the serine / threonine phosphatase, PP2A, in regulating the effects of the two tyrosine kinase inhibitors, imatinib and nilotinib, on glioma signalling and motility. PP2A has been reported to interact with p130Cas and it has been proposed that PP2A plays a role in regulating the cell cycle through de-phosphorylation of p130Cas (Ito et al., 2000). Although PP2A has been reported to de-phosphorylate p130Cas on serine residues (Yokoyama and Miller, 2001b), there are no reports of how PP2A activity affects p130Cas tyrosine phosphorylation. But it has been recently reported that long-term 24 hour treatment with nilotinib leads to PP2A inactivation in human HCC cells (Yu et al., 2013). Consistent with the possibility that inactivation of PP2A by imatinib or nilotinib treatment results in enhanced p130Cas, FAK and PXN tyrosine phosphorylation, treatment with the PP2A inhibitor, OA, significantly increased basal tyrosine phosphorylation of these molecules. If imatinib- and nilotinib-induced increased tyrosine phosphorylation is completely dependent on PP2A activity, then treatment with OA should result in similar increases in tyrosine phosphorylation. However, if the concentration of OA used was not sufficient to fully inhibit PP2A then combined treatment with both OA and drug should have an additive effect over single treatments. To fully understand this effect, dose response curves would need to be performed. Preliminary analysis of potential additive effects based on either imatinib or nilotinib alone, or in combination with OA, suggest that in some cases drug treatments were not additive, and in other cases they were additive. Nonetheless, the role of PP2A in mediating imatinib and nilotinib effects is confirmed by the results obtained from the treatment with FTY720. Pharmacological activation of PP2A, using FTY720, strongly inhibited both the increased tyrosine phosphorylation of p130Cas, FAK and PXN and radial spheroid invasion stimulated by imatinib and nilotinib. It is proposed that PP2A activity is playing a key role in modulating the effects of imatinib and nilotinib via regulation of tyrosine phosphorylation of p130Cas, FAK and PXN.

PP2A is one of the few serine/threonine-specific phosphatases in the cell and one of the most abundant enzymes, accounting for up to 1% of total cellular protein in some tissues. PP2A plays an important role in development, cell proliferation, cell death, cell mobility, the control of the cell cycle, and the regulation of numerous signalling pathways (Shi, 2009). PP2A is an important tumour suppressor, regulating the activity of at least 50 different kinases and has been shown to antagonise the actions of Ras (Janssens et al., 2005, Mumby, 2007). Given the results here, it is proposed that modulating PP2A activity could provide an approach to improving the clinical outcome of imatinib and nilotinib. Stimulating PP2A activity could boost efficacy and could help circumvent the development of resistance to these drugs in CML and other cancers for which they are used.

It has been shown that in BCR-ABL-transformed cells and CML blast crisis progenitors, the phosphatase activity of PP2A is inhibited by the BCR-ABL-induced expression of the PP2A inhibitor SET. PP2A activation resulted in growth suppression and enhanced apoptosis, and leukaemogenesis was found to be dependent on inactivation of PP2A (Neviani et al., 2005). FTY720 induces toxic effects in primary leukaemic cells from AML patients and in AML cell lines. (Yang et al., 2012). Results presented here support the role of PP2A as a tumour suppressor and suggest that pharmacological modulation of PP2A may help circumvent the development of resistance to imatinib and nilotinib. Findings highlight that specific molecular effects of imatinib and nilotinib in different cell types are not well defined.

6 Imatinib and nilotinib effects on serine phosphorylation

6.1 Imatinib and nilotinib stimulate serine phosphorylation of p130Cas, FAK and PXN

Tyrosine phosphorylation has emerged as a critical component of cellular regulation, predominantly because tyrosine kinases have been found to initiate many signalling pathways. However, signal transduction involves reversible phosphorylation of proteins not only on tyrosine residues, but also on serine / threonine residues (Hunter, 1995). Cell transformation can result from deregulated serine kinase activity, and serine kinases can also initiate signalling pathways. The identification of bifunctional protein kinases further complicates the interplay of tyrosine and serine / threonine phosphorylation; for example, ERK autophosphorylates itself on tyrosine and threonine residues, but phosphorylates substrates on serine or threonine residues (Posada and Cooper, 1992).

The tyrosine phosphorylation of p130Cas, FAK and PXN in mediating cell motility has been widely studied. Src directly phosphorylates p130Cas, FAK and PXN at tyrosine residues in response to integrin activation and growth factor stimulation (Sharma and Mayer, 2008, Barrett et al., 2013). FAK is activated by growth factors, which leads to autophosphorylation at its tyrosine residue Y397 (Nakamura et al., 2001). Src phosphorylation of FAK tyrosine residue Y861 enhances FAK binding to p130Cas (Mitra et al., 2005). Tyrosine phosphorylation of PXN at tyrosine residues Y31 and Y118 occurs

during the formation of focal adhesions and generates two functional SH2-binding sites for members of the Crk family of SH2-SH3 adaptor proteins (Turner, 2000a, Petit et al., 2000). By contrast, serine-specific phosphorylation of p130Cas, FAK and PXN is not widely reported in the literature, nor has the involvement of serine phosphorylation in mediating motility and other cell functions been widely investigated. Several studies, however, suggest that not only tyrosine phosphorylation, but also serine phosphorylation, is important in driving the activity of p130Cas, FAK and PXN. Serine phosphorylation of p130Cas has been shown to be required for the coordinated disassembly of focal adhesion complexes during mitosis, and is associated with simultaneous serine phosphorylation of FAK and PXN (Yamakita et al., 1999). Serine phosphorylation of p130Cas correlates with oestrogen resistance and invasiveness of human breast cancer cells (Makkinje et al., 2009)

The interplay between tyrosine phosphorylation and serine phosphorylation of p130Cas has not been widely studied. The NRTK Src was recently found to mediate serine phosphorylation of p130Cas at residue S639 in an *in vivo* feline model of pressure overloaded myocardium. Whereas kinase activity of Src was required for tyrosine phosphorylation of p130Cas at tyrosine residues Y165, Y249 and Y410, kinase inactive Src mutant with intact scaffold domains was able to phosphorylate p130Cas in hamster ovary cells overexpressing p130Cas at a serine residue corresponding to human S639 (Palanisamy et al., 2015). These findings demonstrate that Src can mediate serine phosphorylation of p130Cas at S639, and that this occurs independently of Src kinase activity that is required for tyrosine phosphorylation of the protein.

Previous findings in this thesis show that the serine / threonine, PP2A, regulates the effects of imatinib and nilotinib on glioma cell signalling and motility. Inhibition of PP2A with okadaic acid significantly increased levels of tyrosine phosphorylation of p130Cas, FAK and PXN. Conversely, activation of PP2A with FTY720 strongly inhibited drug-induced tyrosine phosphorylation of the three molecules (**Fig. 5.2.1**), and radial invasion (**Fig. 5.2.3**). Based on the findings that a serine / threonine phosphatase is involved in mediating imatinib- and nilotinib-induced tyrosine phosphorylation of p130Cas, FAK and PXN, the effect of drug treatment on serine phosphorylation of the three proteins was investigated.

There are no commercially available serine-specific p130Cas antibodies. To assess whether imatinib and nilotinib may be affecting serine phosphorylation of p130Cas, cell lysates were treated with imatinib or nilotinib, immunoprecipitated with anti-phosphoserine antibody and blotted for total p130Cas. Findings reveal that 20-minute treatment with 10 μM imatinib or 10 μM nilotinib causes a significant increase in the amount of p130Cas detected (**Fig. 6.1.1**).

FAK contains four serine phosphorylation sites within its C-terminal domain, S722, S843, S846 and S910 (Ma et al., 2001), that are in close proximity to important protein-protein interaction sites, such as the p130Cas binding site (**Fig. 1.2.2.2.1**). Serine phosphorylation at S722 may play a role in modulating FAK binding to p130Cas (Ma et al., 2001), and FAK has been found to undergo Src-dependent S910 phosphorylation, which is important for cell spreading (Chu et al., 2011). The effect of imatinib and nilotinib on FAK serine phosphorylation was investigated by treating cells with imatinib or nilotinib, immunoprecipitating cell lysates with an anti-phosphoserine antibody, and blotting for total FAK. Preliminary findings suggest that more total FAK is detected in response to 20-minute treatment with 10 μM imatinib or 10 μM nilotinib, than in response to control (**Fig. 6.1.2**).

In addition to tyrosine phosphorylation sites, serine phosphorylation sites have also been identified on PXN. Serine residues S188 and S190 were found to be phosphorylated following integrin activation (Bellis et al., 1997). More recently, PXN serine S126 and S130 residues were identified as two major ERK-dependent phosphorylation sites in Raf transformed fibroblasts (Woodrow et al., 2003). Serine residue S126 was subsequently found to be stimulated in response to lipopolysaccharide (LPS) in murine macrophage cells. LPS stimulation was accompanied by cell spreading that was partially inhibited in cells expressing the PXN S126A/ S130A mutant (Cai et al., 2006). LPS is a component of the outer membrane of Gram-negative bacteria that strongly activates macrophage migration from blood vessels into surrounding tissue (Kleveta et al., 2012).

Based on the previous finding that PXN serine phosphorylation mediates macrophage cell spreading, the effect of imatinib and nilotinib treatment on PXN serine phosphorylation was examined using a phospho-specific antibody. Before treatment with imatinib and nilotinib, U87MG cells were treated with LPS for 60-minute and lysates were probed with phospho-specific S126 antibody. Consistent with the literature, LPS treatment led to an increase in

the amount of S126 detected by Western blot (**Appendix Fig. A5**). U87MG cells were subsequently treated with 10 μM imatinib or 10 μM nilotinib for 20 minutes, and cell lysates were probed with phospho S126 antibody. Results indicate that treatment with nilotinib leads to a significant increase in serine phosphorylation of PXN at residue S126 (**Fig. 6.1.3**). Results show a trend towards increased serine phosphorylation with imatinib treatment, although this was not significant.

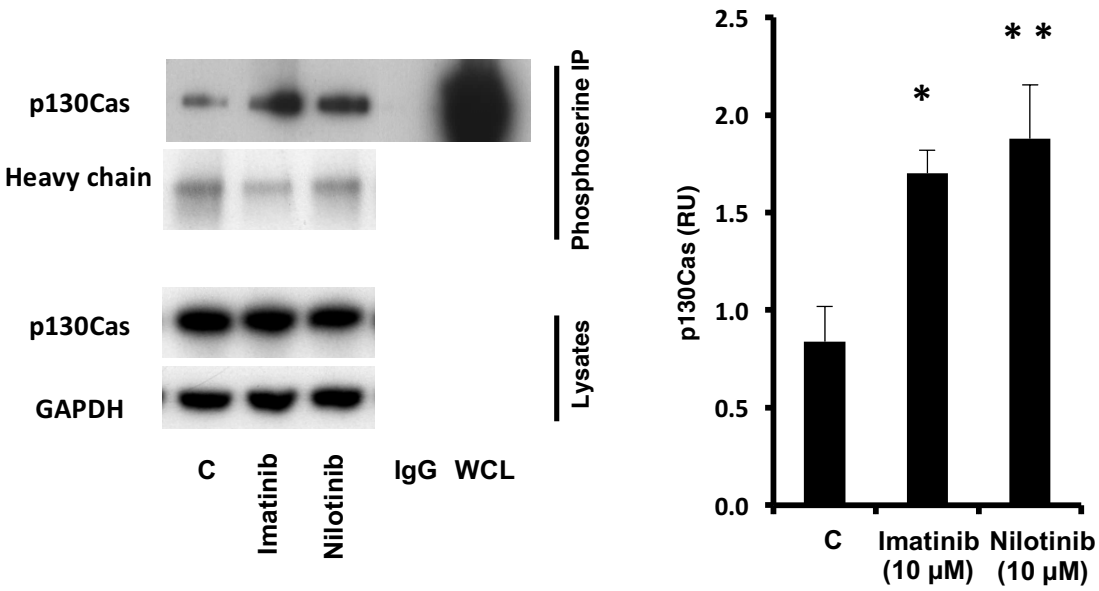


Figure 6.1.1 Imatinib and nilotinib stimulate p130Cas serine phosphorylation in U87MG cells. U87MG cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with either SFM & DMSO vehicle control (C), 10 µM imatinib or 10 µM nilotinib for 20 minutes. Cell lysates were then immunoprecipitated with anti-phosphoserine antibody. Purified complexes and corresponding total cell lysates were separated by SDS-PAGE. IgG control and a whole cell lysate (WCL) were also included. Data from five independent experiments are presented as phosphorylation relative units (RU) (means +/- s.e.m.) normalised to the loading control (the heavy chain); *$p<0.05$, **$p<0.01$.

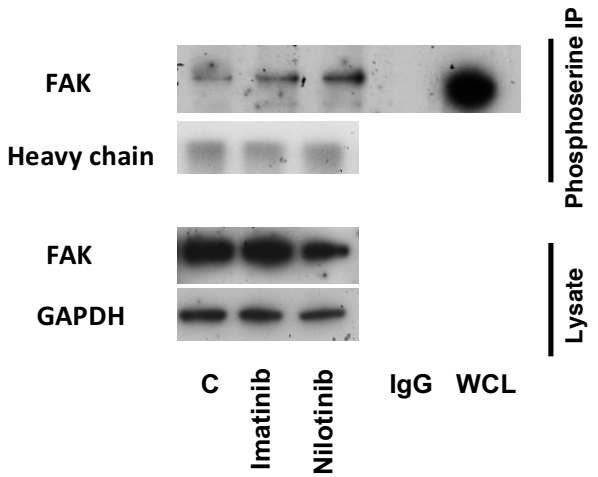


Figure 6.1.2 Imatinib and nilotinib stimulate FAK serine phosphorylation in U87MG cells. U87MG cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with either SFM & DMSO vehicle control (C), 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then immunoprecipitated with anti-phosphoserine antibody. Purified complexes and corresponding total cell lysates were separated by SDS-PAGE. IgG control and a whole cell lysate (WCL) were also included. Blots are representative of *n*=2 experiments.

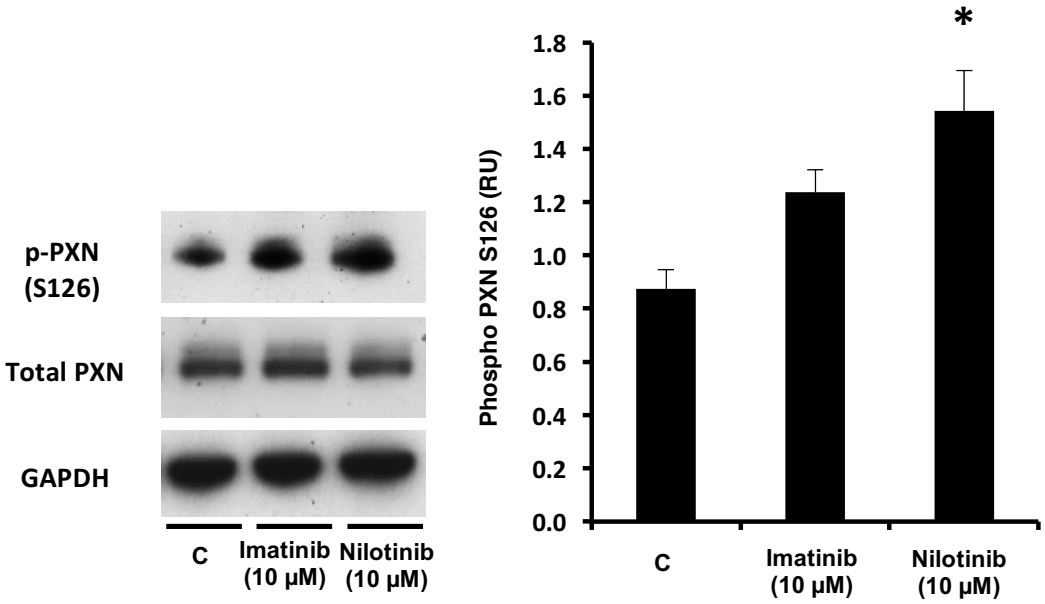


Figure 6.1.3 Imatinib and nilotinib treatment of U87MG cells leads to increased PXN serine phosphorylation. U87MG cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with either SFM & DMSO vehicle control (C), 10 µM imatinib or 10 µM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Data from three independent experiments are presented as phosphorylation relative units (RU) (means +/- s.e.m.) normalised to total PXN protein levels; *$p<0.05$ compared to control.

6.2 Discussion

Findings reveal that imatinib and nilotinib treatment results in increased serine phosphorylation of p130Cas. Furthermore, nilotinib treatment results in a significant increase in serine phosphorylation of PXN S126 residue. Preliminary findings suggest that imatinib and nilotinib may also be stimulating serine phosphorylation of FAK. This is the first study that implicates tyrosine kinase inhibitors in driving serine phosphorylation. Findings in Chapter 5 identify serine / threonine PP2A activity in mediating imatinib- and nilotinib-induced tyrosine phosphorylation. It could be speculated that PP2A modulates serine phosphorylation of p130Cas, FAK and/or PXN, a possibility that would require further work to confirm. It has previously been shown that PP2A associates with p130Cas in mouse embryo fibroblasts. Furthermore, p130Cas immunoprecipitates treated with OA showed an increase in serine phosphorylated p130Cas (Yokoyama and Miller, 2001b). In this study PP2A was found to associate with p130Cas in U87MG cells (**Fig. 5.2.2**). In order to investigate whether PP2A mediates p130Cas serine phosphorylation in U87MG, cells could be pre-incubated with FTY720 prior to treatment with imatinib and nilotinib. Cell lysates immunoprecipitated with anti-phosphoserine antibody could be probed for p130Cas. If PP2A is implicated in imatinib- and nilotinib-induced serine phosphorylation of p130Cas, treatment with FTY720 would stimulate PP2A activity and should inhibit drug-induced phosphorylation. This would mean that less total p130Cas is detected by Western blot. Similar experiments could be performed to look at the effect of modulating PP2A activity on FAK and PXN serine phosphorylation.

Both tyrosine and serine phosphorylation of p130Cas are implicated in modulating the activity of p130Cas. Src scaffold activity mediates serine phosphorylation of p130Cas at serine residue S639, distinct from Src kinase-dependent tyrosine phosphorylation of p130Cas (Palanisamy et al., 2015). BCAR3-mediated serine phosphorylation of p130Cas in breast cancer cells has also been identified and is associated with an aggressive phenotype. BCAR3 is a member of the NSP family of adaptor proteins, which bind Crk-associated substrate (CAS) family of adaptor proteins, including p130Cas, to link CAS proteins to cell surface receptors. NSP proteins affect p130Cas protein expression levels, phosphorylation state, and subcellular localization, affecting migration and invasion. Augmented motility and aggressiveness of breast cancer cells is dependent on the association of BCAR3 with

p130Cas (Wallez et al., 2012, Makkinje et al., 2012). BCAR3 has been found to mediate p130Cas serine phosphorylation at serine residues S139, S437 and S639 in breast cancer cells. p130Cas serine phosphorylation was found to accumulate over several hours after adhesion of breast cancer cells to fibronectin and was dependent on BCAR3 expression. BCAR3 knockdown altered p130Cas localization and inhibited the formation of cellular projections (Makkinje et al., 2009). The BCAR3-mediated serine phosphorylation of p130Cas identified in breast cancer cells is distinct from the rapid FAK- and Src-mediated tyrosine phosphorylation of p130Cas.

Data from this study provide evidence that imatinib and nilotinib modulate both tyrosine and serine phosphorylation of motility-related proteins. The potential involvement of serine phosphorylation of p130Cas, FAK and PXN in modulating U87MG motility, and the potential involvement of PP2A in mediating serine phosphorylation, warrants further investigation.

7 Clinical relevance

7.1 Imatinib and nilotinib treatment of human-derived GBM stem cells leads to increased p130Cas, FAK and PXN tyrosine phosphorylation and increased three-dimensional migration

Cancer cell lines have enabled the study of GBM tumour biology for over 30 years, and have provided useful preclinical models for the screening of therapeutics. However, there are limitations to cell models as the phenotypic characteristics and genetic abnormalities found within repeatedly passaged cell lines often do not resemble the corresponding primary tumour. The serum-containing medium in which cells are cultured alter their genomes and transcriptomes. Furthermore, tumours formed from these cells in mice xenograft models do not develop the typical morphological features of GBM, such as diffuse infiltration into surrounding tissue (Xie et al., 2015, Lee et al., 2006).

There is now a body of evidence that exists to suggest that a sub-population of cancer stem cells exist within brain tumours that are capable of initiating and sustaining tumour growth *in vivo* (Wang and Dick, 2005). Glioma stem cells (GSCs) are now thought to be responsible for treatment resistance, and are implicated in recurrence after resection (Persano et al., 2013). Therefore, the incorporation of GSCs into research *in vitro* would provide a more biologically relevant model system for the study of GBM.

Research has shown that GSCs can be readily cultured as spheres, utilising the same conditions as for the isolation of normal neural stem cells (NSCs). NSCs were originally isolated using serum-free medium without the addition of substrate, and cultured on tissue culture plastic. The subsequent addition of EGF stimulated the proliferation of NSCs, resulting in the formation of clonal spheres, referred to as "neurospheres", that show intraclonal heterogeneity in the expression of neural lineage-specific proteins, the defining characteristic of NSCs (Rahman et al., 2015). This protocol, referred to as the "neurosphere assay", can be applied for the isolation and expansion of GSCs (Ignatova et al., 2002).

The neurosphere assay has become the standard for maintaining GSCs in culture, providing a more clinically relevant model for the study of GBM biology and the effects of therapeutics on GBM. We were kindly given GSCs by Professor Sebastian Brandner at the UCL Institute of Neurology, which were derived from patient biopsies using the neurosphere assay protocol as described in Materials and Methods. The relevance of previous findings using GBM cell lines as outlined in Chapter 3 were assessed by investigating the effects of imatinib and nilotinib on GSCs isolated from patient biopsies. Treatment of patient-derived GSCs with 10 μM imatinib or 10 μM nilotinib led to a significant increase in tyrosine phosphorylation of p130Cas, FAK and PXN (**Fig. 7.1.1A**). Furthermore, treatment of spheroids generated from the GSC line, and two additional GSC lines, with 10 μM imatinib or 1 μM nilotinib led to a significant increase in 3D radial invasion (**Fig. 7.1.1B**).

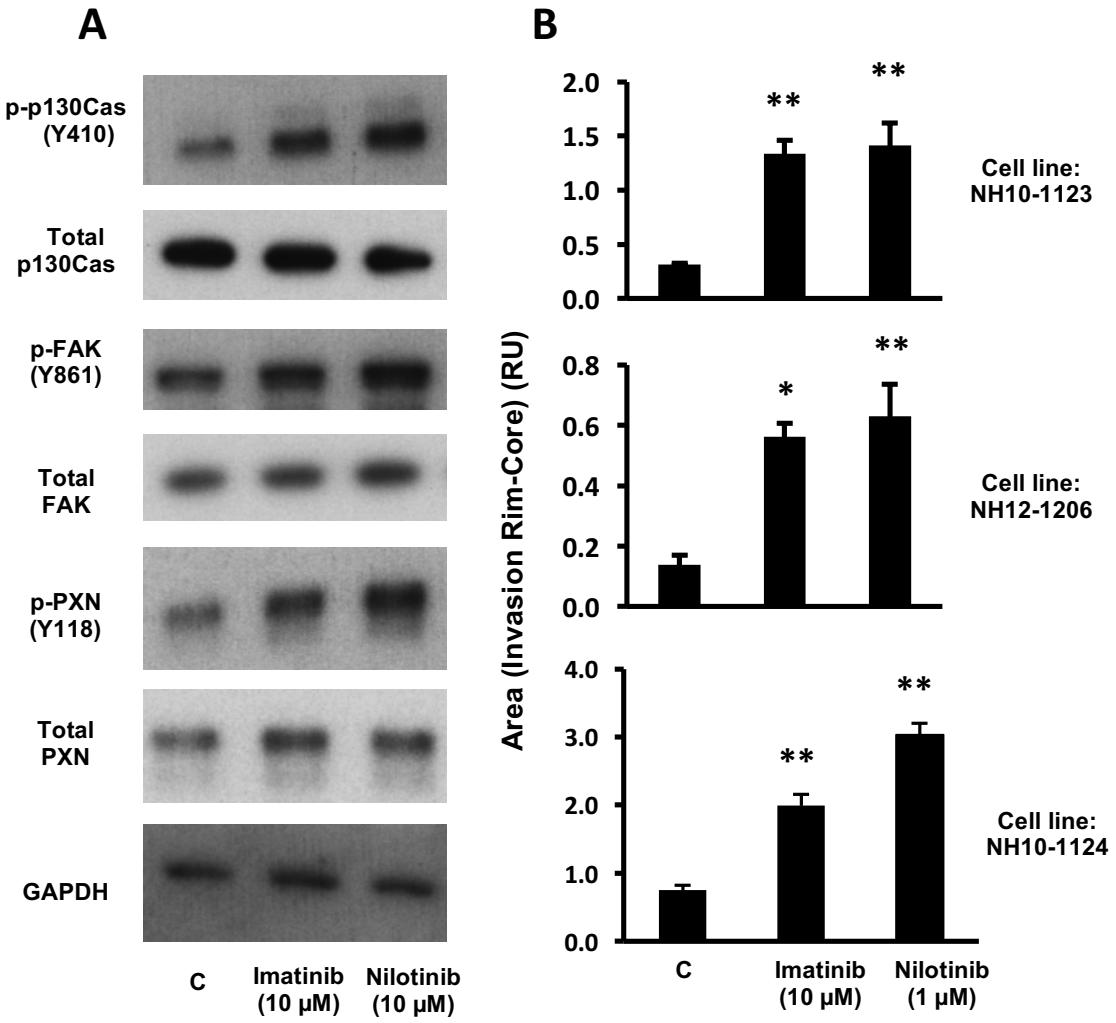


Figure 7.1.1 Imatinib and nilotinib treatment of stem cells from human GBM biopsies leads to increased tyrosine phosphorylation and increased 3D radial invasion. (**A**) Stem cells established from a human GBM biopsy (cell line: NH10-1124) (~80% confluent) were incubated in SFM for ~18hr prior to treatment with either SFM & DMSO vehicle control (C), 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Data is representative of *n*=3 experiments. (**B**) Spheroids derived from the indicated stem cell lines established from human GBM biopsies were imbedded in a collagen gel and incubated in either SFM & DMSO vehicle control (C), or 10 μM imatinib or 1 μM nilotinib for an additional 48 hours. Spheroids were fixed in 4% PFA and invasion was determined by measuring the area corresponding to the invasion rim minus the area of the core for at least 3 different spheroids per condition. Data from three independent experiments are presented as relative area units (RU) (means +/- s.e.m.). *p<0.05, **p<0.01 compared to control (C).

7.2 Imatinib and nilotinib treatment of human gastrointestinal stromal cells leads to increased p130Cas, FAK and PXN tyrosine phosphorylation

Gastrointestinal stromal tumour (GIST) is the most common mesenchymal tumour of the digestive tract. The most common early event in oncogenesis is gain-of-function mutations in KIT or PDGFRA genes which are present in the majority of GISTs and render the proteins constitutively activated. Imatinib was approved for GIST in 2008, and achieves a partial response or stable disease in most patients, with the response lasting more than 5 years in over 20% of patients. However, complete responses are rare, and half of patients develop disease progression after two years. The response to imatinib is dependent on the presence and variety of KIT or PDGFRA gain-of-function mutations (Joensuu, 2007, Guo et al., 2009). Nilotinib has not been approved for GIST, and instead the second-line therapy for imatinib-resistant patients is sunitinib, a TKI that is able to inhibit a proportion of imatinib-resistant c-Kit mutants. However, sunitinib only extends survival by a median of 6 months, as compared to placebo following discontinuation of imatinib (Joensuu, 2007).

To assess whether imatinib and nilotinib may be driving adverse events in GIST, the effect of drug treatment on two GIST cell lines was examined. Currently, there is no suitable GIST model for the study of drug resistance or metastasis, and research relies largely on GIST cells lines obtained from patients. We were kindly provided imatinib-sensitive GIST882 and imatinib-resistant GIST48 cells by Professor Bart Vanhaesebroeck at the UCL Cancer Institute. The cell lines were initially obtained from patients undergoing surgical resection following their consent. The imatinib-sensitive GIST882 human cell line was established from an untreated GIST with a primary homozygous missense mutation in KIT exon 13. The cells do not express wildtype c-Kit, and instead the established from a GIST patient whose cancer had progressed after an initial response to imatinib. GIST48 cells have a mutation in the activation loop of KIT that renders the protein imatinib-resistant (Bauer et al., 2006).

Imatinib-sensitive GIST882 cells were treated with 10 μM imatinib and 10 μM nilotinib. Preliminary findings suggest that 20-minute treatment with 10 μM nilotinib may lead to an increase in tyrosine phosphorylation of p130Cas and FAK. 20-minute treatment with 10 μM imatinib did not have an effect on tyrosine phosphorylation of p130Cas, FAK or PXN. Both 10 μM imatinib and 10 μM nilotinib treatment resulted in reduced phosphorylation of ERK compared to control (**Fig. 7.2.1A**).

A preliminary dose-dependency experiment suggests that whereas treatment with 10 μM imatinib has no effect on tyrosine phosphorylation, treatment with 20 μM imatinib may be increasing tyrosine phosphorylation of p130Cas. Preliminary findings suggest that nilotinib treatment causes an increase in tyrosine phosphorylation of p130Cas in a dose-dependent manner, with nilotinib concentration as low as 1 μM leading to increased phosphorylation. The results for FAK and PXN tyrosine phosphorylation are difficult to interpret requiring further investigation, with possible increases in tyrosine phosphorylation of FAK and PXN upon treatment with 20 μM nilotinib. Results suggest that treatment with imatinib as low as 1 μM, and nilotinib as low as 10 μM, inhibits the phosphorylation of ERK (**Fig. 7.2.1B**). Unfortunately, the GIST882 cells stopped responding to cell culture conditions required for proliferation. Several unsuccessful attempts were made to raise cells from freeze-down vials. We contacted our collaborators who advised that this is an inherent problem of human-derived GIST cells. It was not possible to perform additional experiments using GIST882 cells to better identify the effects of imatinib and nilotinib treatment on tyrosine phosphorylation, nor was it possible to perform functional spheroid assays using these cells.

However, the imatinib-resistant GIST48 cells responded to cell culture conditions and proliferated. Subsequent experiments looking at the effect of imatinib and nilotinib on GIST were performed using these cells. Based on the finding that 20 μM imatinib may be having an effect on tyrosine phosphorylation of p130Cas in GIST882 cells (**Fig. 7.2.1B**), imatinib-resistant GIST48 cells were treated with 20 μM imatinib or 10 μM nilotinib for 20 minutes. Treatment with both imatinib and nilotinib led to a significant increase in tyrosine phosphorylation of p130Cas, FAK and PXN. Interestingly, imatinib and nilotinib treatment did not affect levels of phosphorylated ERK (**Fig. 7.2.2**). This is in contrast to imatinib-sensitive GIST882 cells where imatinib and nilotinib treatment leads to a decrease in the phosphorylation of ERK (**Fig. 7.2.1**).

The effect of imatinib and nilotinib treatment on radial invasion in GIST48 cells was examined. GIST48 spheroids were generated and embedded in collagen plugs supplemented with serum free medium containing either DMSO (control), 10 μM imatinib, 20 μM imatinib or 1 μM nilotinib. Treatment with either imatinib or nilotinib alone for 48 hours resulted in a significant increase in radial invasion compared to treatment with vehicle control (**Fig. 7.2.3**).

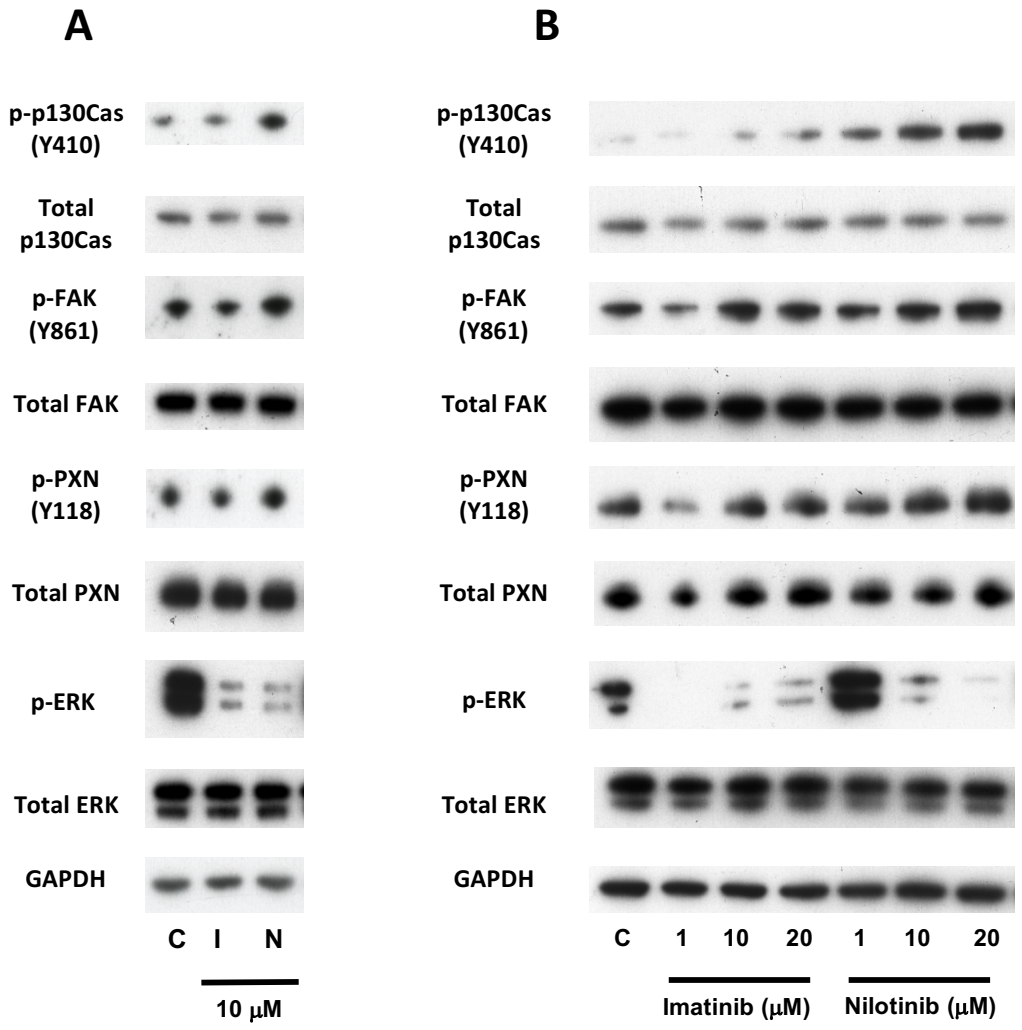


Figure 7.2.1 Imatinib and nilotinib treatment of imatinib-sensitive GIST882 cells. GIST882 cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with (**A**) SFM & DMSO vehicle control (C), 10 μM imatinib (I) or 10 μM nilotinib (N) for 20 minutes; (**B**) SFM & DMSO vehicle control (C), or imatinib or nilotinib at indicated concentrations for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Blots for (**A**) are representative of *n*=2 experiments.

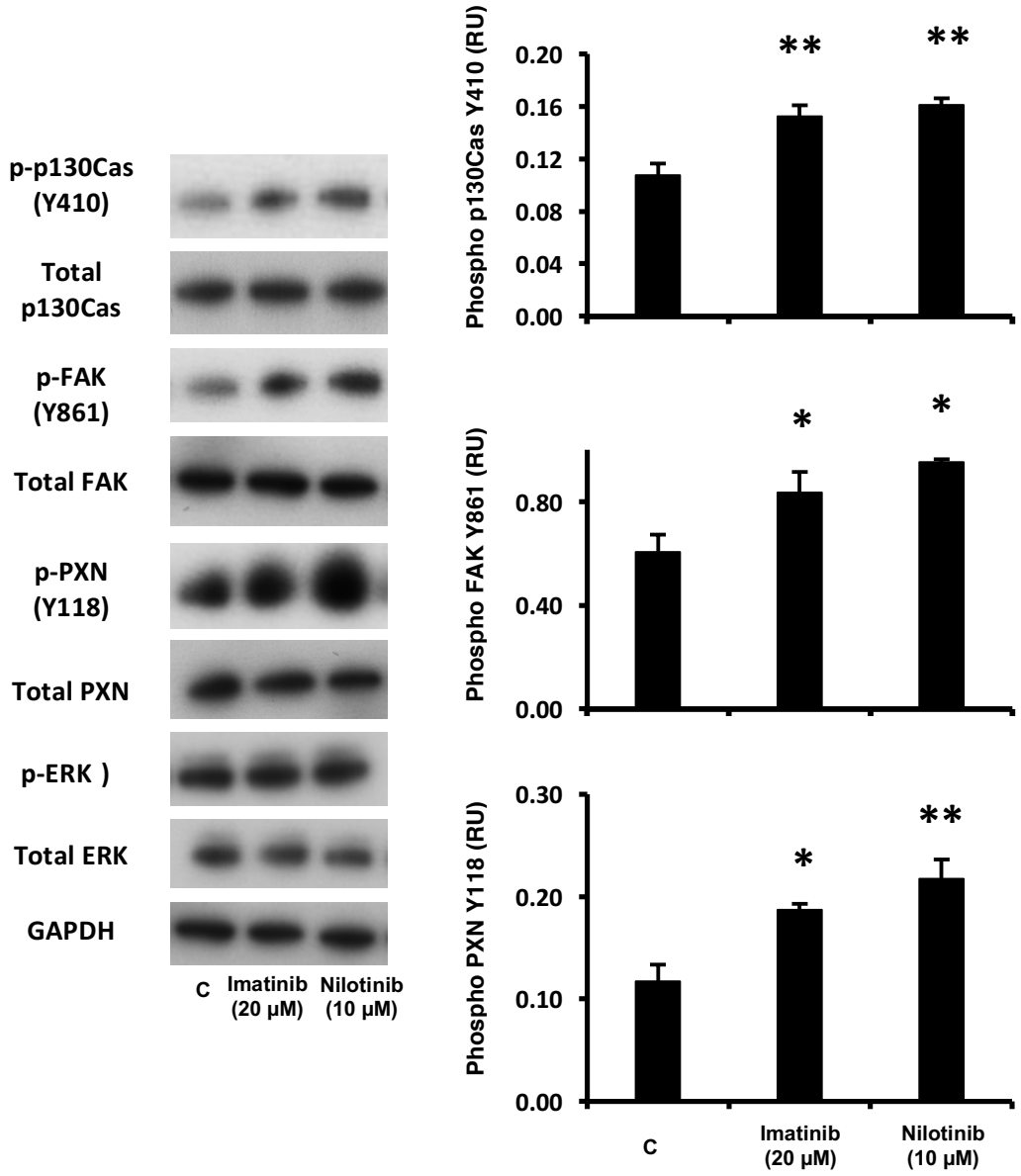


Figure 7.2.2 Imatinib and nilotinib treatment of imatinib-resistant GIST48 cells leads to increased p130Cas, FAK and PXN tyrosine phosphorylation. GIST48 cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with either SFM & DMSO vehicle control (C), 20 µM imatinib or 10 µM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Data from three independent experiments are presented as phosphorylation relative units (RU) (means +/- s.e.m.) normalised to total protein levels; *p<0.05, **p<0.01 compared to control (C).

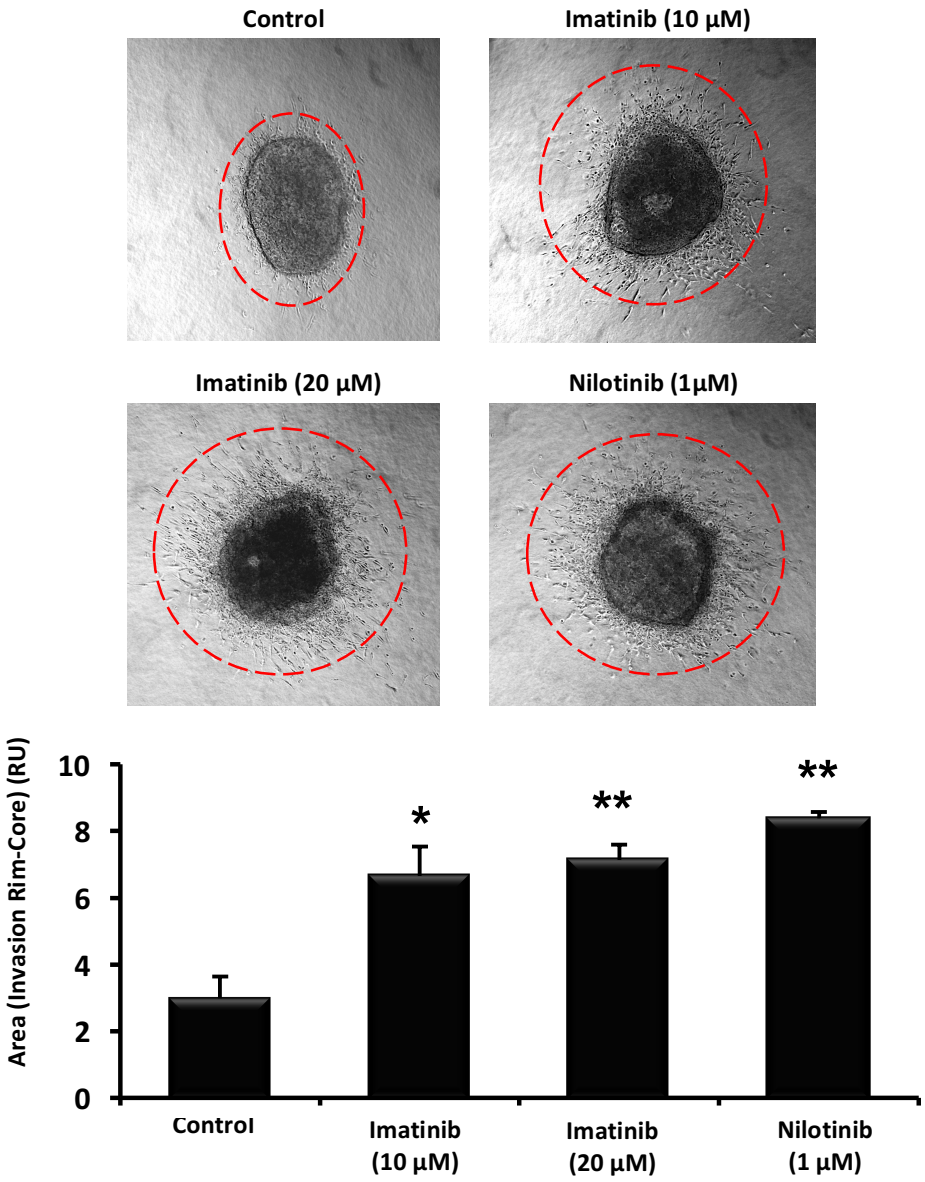


Figure 7.2.3 Imatinib and nilotinib treatment of human GIST cells leads to increased 3D radial invasion. Equal amounts of GIST48 cells were used to generate spheroids as described in Materials and Methods. 24 hours after spheroid production, spheroids were imbedded in a collagen gel and incubated in SFM supplemented with vehicle (control), 10 µM imatinib, 20 µM imatinib or 1 µM nilotinib for 48 hours. Spheroids were fixed in 4% PFA and invasion was determined by measuring the area corresponding to the invasion rim minus the area of the core for at least 3 different spheroids per condition. Data from three independent experiments are presented as relative area units (RU) (means +/- s.e.m.); *p<0.05, **p<0.01 compared to control.

7.3 Imatinib and nilotinib treatment of hepatocellular liver carcinoma cells leads to increased p130Cas, FAK and PXN tyrosine phosphorylation

To examine whether increases in tyrosine phosphorylation in response to imatinib and nilotinib are specific to GBM and GIST cancer cells, the effect of drug treatment on hepatocellular carcinoma (HCC) was investigated. HEPG2 cells previously used by our lab were treated with imatinib and nilotinib. Findings reveal that a 20-minute treatment with 10 μM imatinib or 10 μM nilotinib leads to a significant increase in tyrosine phosphorylation of p130Cas. Treatment with 10 μM nilotinib also leads to a significant increase in tyrosine phosphorylation of FAK and PXN (**Fig. 7.3.1**). These results show that imatinib- and nilotinib-induced increases in tyrosine phosphorylation are not restricted to GBM and GIST cancer cells.

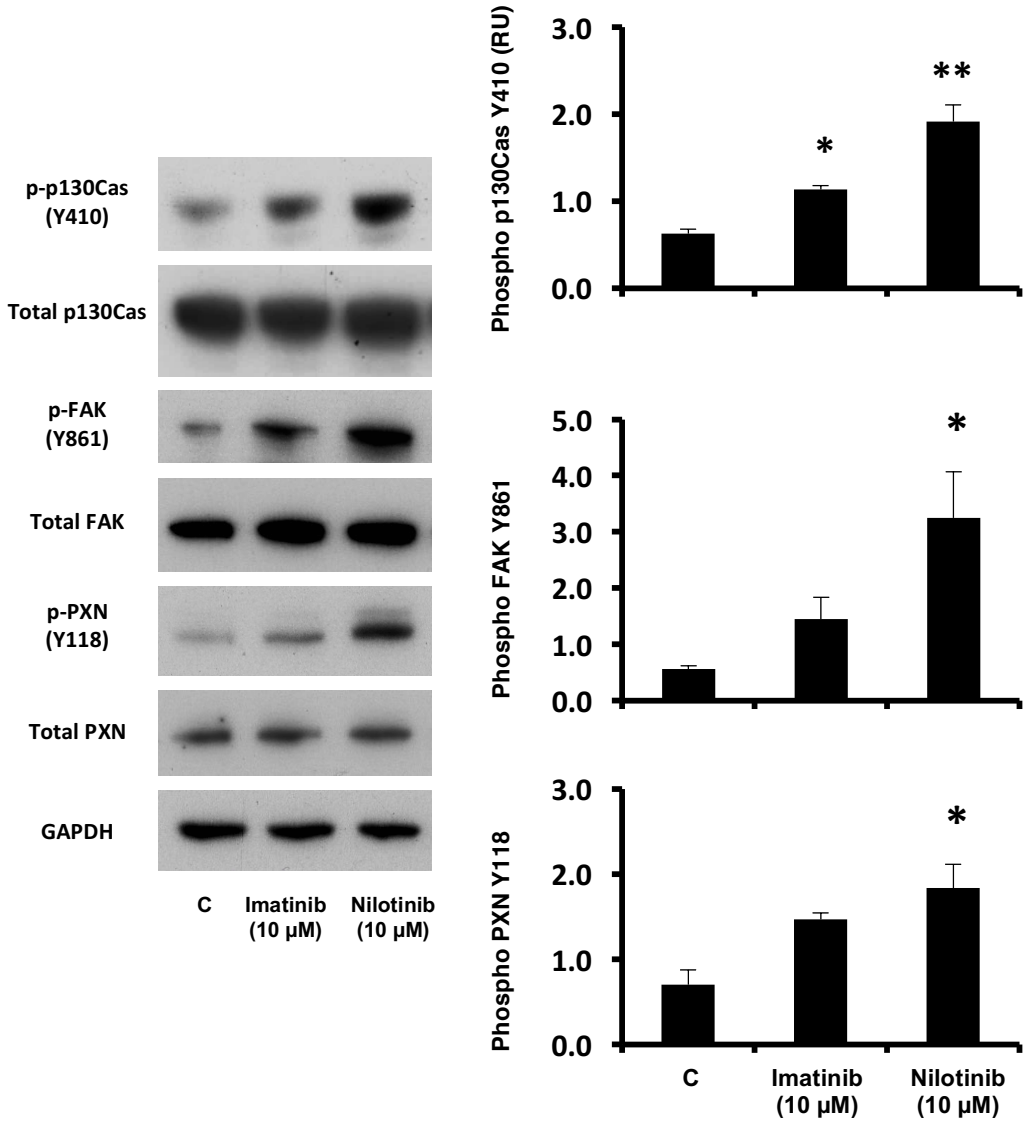


Figure 7.3.1 Imatinib and nilotinib treatment of human liver carcinoma HEPG2 cells leads to increased p130Cas, FAK and PXN tyrosine phosphorylation. HEPG2 cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with vehicle control (C), or 10 µM imatinib or 10 µM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Data from three independent experiments are presented as relative units (RU) (means +/- s.e.m.); *p<0.05, **p<0.01 compared to control.

7.4 Imatinib and nilotinib treatment of non-cancer cells leads to increased p130Cas, FAK and PXN tyrosine phosphorylation

Imatinib and nilotinib are described as well-tolerated drugs that cause few major adverse effects, with common reported adverse effects restricted largely to skin rash, diarrhoea, headache, nausea and fatigue (Druker et al., 2006, Quintas-Cardama et al., 2010, Waller, 2010). However, adverse cardiovascular effects in response to imatinib or nilotinib treatment have been reported in several independent studies, more commonly identified in patients receiving higher doses (600-800 mg/day). One study identified imatinib treatment as a cause of left ventricular dysfunction in CML patients treated with 400-800 mg/day imatinib. Healthy mice treated with imatinib at doses comparable to those in the clinic also developed significant deterioration in contractile function and moderate left ventricular dilation after 3-4 weeks of treatment. Moreover, the study found that imatinib caused cell death in rat ventricular myocytes (Kerkela et al., 2006). In one study looking at 24 CML patients treated with nilotinib, adverse vascular events occurred in 8 of the 24 patients (~33%). Three patients developed rapidly progressive peripheral arterial disease (PAD), requiring repeated angioplasty and/or multiple surgeries within a few months. An additional one less severe case of PAD was identified in the remainder of the cohort, as well as one myocardial infarction, one spinal infarction and one haematoma (Aichberger et al., 2011). Adverse vascular events, including accelerated atherosclerosis, PAD and coronary artery disease, have also been documented in other studies (Tefferi and Letendre, 2011, Coon et al., 2013).

To assess whether the activation of p130Cas, FAK and PXN by imatinib and nilotinib is a general effect or a disease-specific effect, primary non-tumour human umbilical vein endothelial cells (HUVECs) and human coronary artery smooth muscle cells (HCASMCs) were treated with imatinib and nilotinib. These experiments were performed by other members of our lab, Dr. Ian Evans and Miss Lisa Im (both at the Centre of Cardiovascular Biology and Medicine, UCL). Our lab has previously shown that VEGF stimulates tyrosine phosphorylation of p130Cas Y410 and FAK Y397 in HUVECs (Evans et al., 2011). HUVECs were treated with 10 μM imatinib or 10 μM nilotinib followed by incubation with

VEGF or vehicle control. Preliminary findings suggest that 10 μM imatinib and 10 μM nilotinib treatment of HUVECs leads to a rapid increase in tyrosine phosphorylation of p130Cas at Y410, FAK at its activating residue Y397, and PXN at Y118 (**Appendix Figure A6A**). Even in the absence of VEGF stimulation, 20-minute treatment with 10 μM imatinib or 10 μM nilotinib resulted in an increase in tyrosine phosphorylation of all three residues.

Our lab has previously shown that PDGF stimulation of HCASMCs leads to increased tyrosine phosphorylation of p130Cas (Pellet-Many et al., 2011). HCASMCs were treated with nilotinib for 30 minutes followed by incubation with PDGF or vehicle control. Preliminary findings suggest that treatment with1 μM, 5 μM or 10 μM nilotinib leads to an increase in tyrosine phosphorylation of p130Cas, FAK, and PXN, even in the absence of PDGF (**Appendix Figure A6B**). The effects of imatinib on p130Cas, FAK and PXN in HCASMCs were inconclusive and are currently being investigated further in our lab.

7.5 Discussion

Only a minority population of brain tumour cancer stem cells have the ability to self-renew, and these glioma stem cells are thought to drive tumour growth and spread. An important finding of this study is that imatinib and nilotinib treatment of GBM stem cells derived from patient biopsies led to increased tyrosine phosphorylation of p130Cas, FAK and PXN. Imatinib and nilotinib were also found to potently increase 3D radial spheroid invasion. These findings validate the clinical significance of results identified in U87MG, U118MG and U251MG glioma cell lines. It can be concluded that imatinib and nilotinib stimulate pro-invasive activity in a disease relevant model, providing evidence for the first time of why imatinib may have failed clinical trials for GBM.

The effects of imatinib and nilotinib treatment in imatinib-sensitive GIST882 cells were inconclusive. Preliminary findings suggest that imatinib at 20 μM causes an increase in tyrosine phosphorylation of p130Cas. Preliminary findings also suggest that nilotinib induces as stronger activation of tyrosine phosphorylation of p130Cas than imatinib, with nilotinib exerting effects in a dose-dependent manner. Results suggest that nilotinib may cause increases in FAK and PXN tyrosine phosphorylation, although this requires further investigation. In the future an effort should be made to identify a new source of imatinib-sensitive GIST cells that can be cultured. Importantly, imatinib and nilotinib were found to stimulate tyrosine phosphorylation in imatinib-resistant GIST48 cells. Both drugs were also found to stimulate 3D radial invasion in GIST48 cells. Imatinib and nilotinib were found to induce a stronger effect on tyrosine phosphorylation in the imatinib-resistant cells compared to the imatinib-sensitive cells, suggesting that the p130Cas, FAK and PXN pathways may be activated in response to the development of resistance to imatinib. Findings here provide evidence that imatinib and nilotinib treatment cause undesirable adverse effects in GIST, a cancer for which imatinib is approved. Only a small number of GIST patients (10-20%) exhibit primary resistance to imatinib. However, most responding patients develop secondary resistance after a median of two years (Bauer et al., 2007). The activation of the motility-related proteins in GIST48, and possibly GIST882, suggest that p130Cas, FAK, and PXN activity may contribute to resistance and/or tumorigenesis, and that targeting this pathway in GIST patients may prove therapeutically beneficial.

Imatinib and nilotinib caused a reduction in phosphorylated ERK in the GIST882 cells, but not the imatinib-resistant GIST48 cells. These findings implicate the c-Kit receptor in driving downstream activation of ERK. In the imatinib-sensitive cells inhibition of c-Kit leads to inhibition of ERK. The c-Kit mutation in the GIST48 cells renders the receptor unresponsive to imatinib and it subsequently continues to activate the ERK pathway. These findings are consistent with previous findings that the ERK pathway is activated in untreated GISTs and that imatinib treatment causes a reduction in phosphorylated ERK, as identified in patient-derived imatinib-treated GIST biopsies compared to biopsies from untreated patients (Heinrich et al., 2006). These findings support the targeting of downstream signalling proteins as an approach to tackling imatinib resistance. Indeed, additive effects have been observed in GIST cells using imatinib together with the MEK inhibitor U0126 (Bauer et al., 2007).

Findings here are consistent with previous findings by *Rossi et al.* that show increased tyrosine phosphorylation of p130Cas (Y410) and FAK (Y861) in response to imatinib treatment in a mouse model of GIST (Rossi et al., 2010). The researchers propose that targeting FAK and members of the Src family kinases, or their downstream effectors in addition to imatinib would provide added therapeutic benefit. Given that first and second generation drugs are not always effective, an alternative strategy to circumvent TKI-resistance would be to target downstream pathways critical for transformation. Results of this project endorse the idea of a combinational approach and suggest that targeting FAK in conjunction with imatinib may add therapeutic benefit for the treatment of GIST.

The ability of imatinib and nilotinib to induce tyrosine phosphorylation is further underscored by the finding that treatment with either drug stimulates rapid phosphorylation of p130Cas in HEPG2 cells. Nilotinib treatment also led to a rapid increase in tyrosine phosphorylation of FAK and PXN. Results highlight the necessity of screening TKIs of interest across different types of cancers. Importantly, imatinib has failed a phase II clinical study looking at the efficacy and safety of imatinib in patients with unresectable HCC. 15 patients with HCC were enrolled and it was found that although single-agent imatinib was well-tolerated by all patients, imatinib did not show any clinical efficacy for the treatment of HCC (Lin et al., 2008).

Importantly, imatinib and nilotinib were found to stimulate tyrosine phosphorylation in non-tumour cell lines suggesting that the drugs exert general effects that are not restricted to disease. Preliminary findings suggest that imatinib and nilotinib stimulate tyrosine phosphorylation of p130Cas, FAK and PXN in HUVECs. Endothelial cells lining the endothelium cover the inner surface of blood vessels and separate blood from tissues. The endothelium also regulates vascular smooth muscle cells, regulating relaxation / contraction, and therefore vasodilatation / vasoconstriction. Endothelial dysfunction contributes to atherosclerotic disease (Esper et al., 2006). The findings that imatinib and nilotinib may stimulate p130Cas, FAK and PXN phosphorylation in endothelial cells suggest that the activation of these proteins may contribute to the prevalence of cardiovascular side effects observed in the clinic. This is further supported by preliminary finding that nilotinib treatment leads to increased tyrosine phosphorylation of p130Cas, FAK and PXN in HCASMCs. Activation of the p130Cas, FAK and PXN pathway in smooth muscle may contribute to the development of adverse cardiovascular effects.

8 Concluding discussion

8.1 The development of resistance to tyrosine kinase inhibitors

The approval of the first small-molecule kinase inhibitor, imatinib, in 2001 for the treatment of chronic myeloid leukaemia was a breakthrough in targeted cancer therapy. It provided evidence that you can target an oncoprotein that is the main driver of disease to successfully treat patients without severe side effects. Imatinib has changed the landscape of CML therapy. For patients in chronic phase CML, the rate of complete hematologic response approaches 100%. However, for patients in accelerated phase and blast crises phase, the efficacy of imatinib is significantly lower. And although initial response rates to imatinib are high, the TKI fails in up to 40% of patients with prolonged treatment (Quintas-Cardama et al., 2010, Druker et al., 2006).

Acquired resistance to anticancer therapy remains a significant barrier in reducing the mortality associated with malignancy. The clinical outcome of a TKI is genotype-dependent in regards to both primary and secondary mutations in the drug targets. In the case of CML, imatinib binds with high affinity and specificity to the ATP-binding site within the catalytic domain of BCR-ABL. Response to the drug is maintained by inhibition of the ATP binding domain, preventing signalling downstream of BCR-ABL. Most often acquired resistance occurs as a result of mutations in the BCR-ABL kinase domain which prevent imatinib from binding the ATP-binding site (Quintas-Cardama et al., 2010). Although second-generation nilotinib is effective against many mutations that render BCR-ABL resistant to imatinib, it is not effective against the gatekeeper T315I mutant (Weisberg et al., 2006), which accounts

for 4-20% of mutations that are associated with TKI resistance (Xu et al., 2016). Treatment with the third-generation ponatinib results in a major cytogenetic response in 70% of chronic patients with the T315I mutation. However, the major cytogenetic response drops to 29% for those patients in the blast crisis phase (Cortes et al., 2013).

Acquired resistance to imatinib and nilotinib occurs over months or years, usually as a result of genomic instability which produces sub-clones with mutations in the kinase domain. In addition, non-genetic mechanisms of drug resistance have also been identified. It has recently been suggested that tumour cells can become dependent on non-amplified and non-mutated RTKs to evade targeted treatment, in a phenomenon referred to as "*RTK-switching*". RTK switching has been identified as a mechanism of resistance to EGFR inhibition in GBM. *Akhavan et al.* generated mice xenografted with U87MG cells expressing the EGFRvIII gain-of-function mutation. As expected, erlotinib treatment slowed tumour growth relative to control; however, tumours retained a considerable growth rate despite continued treatment. Inhibition of EGFR was found to promote PDGFRβ upregulation. Immunoblots of tumour lysates revealed upregulation and activation of PDGFRβ in response to pharmacological or genetic inhibition of EGFRvIII. Erlotinib treatment of human-derived EGFRvIII-expressing GBM neurospheres also resulted in upregulation of PDGFRβ expression. A strong inverse correlation between EGFR and PDGFRβ expression was observed in patient glioma tissues, with high levels of EGFR correlating with a low level of PDGFRβ. Erlotinib treatment of human-derived EGFRvIII-expressing GBM neurospheres resulted in upregulation of PDGFRβ expression. This provides evidence that highly active EGFR signalling can negatively regulate PDGFRβ expression, and that inhibition of EGFR signalling results in an RTK switch to PDGFRβ. Importantly, combinational pharmacological inhibition of PDGFR and EGFR in patient-derived GBM neurospheres with high levels of EGFR significantly suppressed tumour cell proliferation (Akhavan et al., 2013). Upregulation of the hepatocyte growth factor pathway has also been documented in response to EGFR kinase inhibitors in lung cancer via focal amplification of the c-Met receptor (Engelman et al., 2007).

Changes in the tumour microenvironment caused by an anticancer drug can affect the response of tumour cells to therapy. One study found that although BRAF-mutant melanoma cells initially respond to the BRAF inhibitor PLX4720, they rapidly develop resistance. PLX4720 leads to matrix remodelling, which promotes integrin β1/FAK/Src

signalling and causes ERK re-activation downstream of BRAF. As expected, combined MEK and BRAF inhibition led to a reduction of ERK signalling. FAK inhibition alone did not lead to reduced ERK activity. Importantly, BRAF inhibition in combination with FAK inhibitor PF-228 prevented the re-activation of ERK in cultured melanoma spheroids embedded in collagen (Hirata et al., 2015). This study highlights that inhibition of a target can generate a drug-tolerant microenvironment prior to the emergence of a genetic cell intrinsic mechanism of drug resistance. This provides an example of how the development of combinational therapies can help circumvent drug-tolerance.

8.2 Combinational therapeutic approach

The preliminary results here highlight the presence of unforeseen effects of imatinib and nilotinib on cellular signalling, implicating the activation of p130Cas and FAK pathways. Imatinib and nilotinib treatment leads to a rapid increase in tyrosine phosphorylation and localisation to the membrane of p130Cas, FAK and PXN. Consistent with increases in tyrosine phosphorylation of motility-related proteins, imatinib and nilotinib stimulate two-dimensional and three-dimensional migration. Findings demonstrate for the first time that imatinib and nilotinib, two tyrosine kinase inhibitors, also stimulate serine phosphorylation of p130Cas, FAK and PXN. Preliminary findings suggest that drug-induced effects may not be dependent on the targets to which the drugs bind to with high specificity. By contrast, results demonstrate that drug-induced effects are dependent on p130Cas, and may be dependent on FAK kinase activity.

A possible mechanism of resistance to imatinib and nilotinib is the upregulation of alternative pathways. As discussed above, studies looking at EGFR inhibition provide evidence of non-genetic adaptive mechanisms of resistance and show how a tumour can become "addicted" to a non-amplified and non-mutated RTK to evade targeted treatment. The study looking at BRAF inhibition in melanoma cells provides evidence that a drug can induce changes to the tumour microenvironment, which results in drug-tolerance. In this context, multiple targets need to be targeted simultaneously. The results here suggest that a combinational approach targeting PP2A and / or FAK in combination with imatinib or nilotinib could have a beneficial therapeutic effect.

Findings in this study implicate the serine / threonine phosphatase, PP2A, in mediating effects of imatinib and nilotinib on glioma signalling and motility. Consistent with the possibility that inactivation of PP2A by imatinib or nilotinib treatment results in tyrosine phosphorylation, pre-treatment with okadaic acid, a PP2A inhibitor, significantly increased basal tyrosine phosphorylation of p130Cas, FAK and PXN. Conversely, pharmacological activation of PP2A with FTY720 strongly reduced both the drug-induced increase in tyrosine phosphorylation of p130Cas, FAK and PXN, and radial invasion. In Ph+ leukaemias, FTY720 promotes the inactivation of BCR-ABL and apoptosis of Ph+ blasts; and FTY720 treatment leads to long-term survival in a mouse model of BCR-ABL-driven

leukaemia (Neviani et al., 2007). Similar findings were observed in AML cells harbouring gain-of- function mutated c-Kit (c-Kit+). Pharmacological activation of PP2A by FTY720 reduced proliferation and induced apoptosis of mutant c-Kit+ cells (Roberts et al., 2010). Findings here support the role of PP2A as a tumour suppressor and suggest that pharmacological modulation of PP2A may add therapeutic benefit when used in conjunction with imatinib/nilotinib.

Given the findings by *Roberts et al.* that FTY720 promotes apoptosis of c-Kit+ leukemia cells, it is possible that reactivation of PP2A may be of therapeutic benefit in other c-Kit+ cancers (Roberts et al., 2010). The effect of FTY720 in GIST has not been widely studied, and based on findings from this project that imatinib and nilotinib also induce tyrosine phosphorylation of p130Cas, FAK and PXN in GIST cells, the effect of FTY720 in GIST cells warrants investigation. Preliminary findings show that FTY720 is significantly cytotoxic in GIST cells (Dumont et al., 2011). However, *Dumont et al.* found that the concentration of FTY720 required to reduce GIST cell viability by 50% at 72 hours in GIST cells was between 2.9 and 3.9 μmol/L, which translates to high oral dosing for patients, and exceeds the concentrations used in clinical trials. The effect of lower doses and long-term treatment of FTY720 should be considered, as well as the potential of a combinational approach alongside imatinib.

FAK inhibition has been found to synergise with nilotinib to reduce Ph+ CML growth *in vivo* (Hu and Slayton, 2014). Findings from this study suggest that FAK inhibition in conjunction with imatinib / nilotinib may also provide therapeutic benefit for the treatment of GBM. Treatment of U87MG cells with the FAK inhibitor PF-228 significantly reduced the imatinib and nilotinib stimulated increases in p130Cas and PXN tyrosine phosphorylation, and drug-induced 3D radial invasion. FAK inhibition with PF-228 also resulted in a significant decrease in the basal level of radial invasion, supporting the role of FAK in mediating migration. Consistent with the possibility that targeting FAK may be beneficial for the treatment of GBM, FAK inhibition has been found to decrease cellular proliferation, adhesion and migration in glioma cells (Shi et al., 2007). Furthermore, FAK inhibition was found to increase survival of mice in an intracranial glioma xenograft model (Liu et al., 2007b).

In addition to combinational therapy that targets several pathways, the therapeutic potential of multiple TKIs targeting a single kinase needs to be considered in the context of acquired resistance resulting from secondary mutations. In CML, acquired resistance to TKIs occurs most often as a result of mutations in the BCR-ABL kinase domain which prevent drug binding (Quintas-Cardama et al., 2010). Nilotinib and dasatinib are approximately ~30-fold and ~325-fold respectively more potent for BCR-ABL than imatinib, and are effective against most imatinib-resistant BCR-ABL mutants except the gate-keeper mutation T315I. An experimental drug, SGX393 (SGX Pharmaceuticals, Inc., San Diego) was found effective against a large range of mutations, including T315I, in cells recovered from patients that are resistant to dasatinib. However, SGX393 was ineffective against several other mutations that nilotinib and dasatinib could inhibit. Importantly, SGX393 in combination with nilotinib or dasatinib was able to entirely abrogate the outgrowth of resistant subclones (O'Hare et al., 2008). The resistance profile of SGX393 complements the resistance profile of nilotinib and dasatinib. Neither of the drugs control all of the mutations; however, SGX393 in combination with either nilotinib or dasatinib was able to suppress resistance. These pre-clinical findings suggest that a rational combination of two kinase inhibitors that have different resistance profiles, could protect against resistance and provide significant therapeutic benefit in CML and GIST.

8.3 Possible mechanisms mediating the observed effects on phosphorylation and motility

Downstream imatinib- and nilotinib-induced tyrosine phosphorylation could result from at least two different mechanisms: activation of an upstream signalling molecule(s) that activate(s) p130Cas, FAK and PXN, or indirect inhibition of one or more PTP that dephosphorylates the proteins. As discussed in Chapter 4, CSK is a non-receptor tyrosine kinase that inhibits Src via phosphorylation of the Src Y527 residue. Direct inhibition of CSK would result in the activation of Src and could promote downstream tyrosine phosphorylation of p130Cas, FAK and PXN. Although preliminary results suggest that CSK is not implicated in mediating imatinib- and nilotinib-induced phosphorylation, direct drug inhibition of an inhibitory kinase has not been ruled out as a possible mechanism.

The second possible mechanism of action is the indirect inhibition of a phosphatase, which under physiological conditions dephosphorylates p130Cas, FAK and PXN. *Zheng et al.* recently found that Ras unexpectedly negatively regulates FAK via activation of the serine / threonine protein kinase ERK and PTP-PEST. Activated Ras induces serine phosphorylation of FAK S910 by ERK, which recruits the enzyme PIN1. PIN1 is an evolutionarily conserved enzyme that binds and isomerises specific phosphorylated motifs in a subset of proteins resulting in their conformational change (Lu and Zhou, 2007). PIN1 binding and isomerisation of FAK causes PTP-PEST to interact with and dephosphorylate FAK at tyrosine residue Y397. Inhibition of FAK mediated by this pathway promotes Ras-induced cell migration, invasion, and metastasis (Zheng et al., 2009). These findings highlight the sequential modification of FAK by Ras involving serine phosphorylation, isomerisation, and tyrosine dephosphorylation, and provides an example of indirect kinase-mediated activation of a PTP.

8.4 Target specificity

It will be important to further investigate which target(s) and downstream signalling pathways are being activated by imatinib and nilotinib. Imatinib and nilotinib are ATP-competitors initially designed to bind to the ATP binding site of BCR-ABL. In addition to BCR-ABL, these drugs target ARG, PDGFR, DDR1 and c-Kit with high specificity (Hantschel et al., 2008). Imatinib and nilotinib bind their targets in the inactive conformation, with the activation loop folded in towards the active site, preventing substrate binding (Zhang et al., 2009). The target(s) via which the observed effects are mediated is presumed to be one or more kinase with an ATP-binding site against which the inhibitors were developed. The ATP nucleotide-binding pocket is located within the catalytic domain of of the kinase. The activation loop is located at the amino-terminal of the ATP-binding site, and controls catalytic activity by switching between different states in a phosphorylation-dependent manner. Both the ATP-binding site and the activation loop are highly conserved regions across most kinases (von Bubnoff et al., 2002). The traditional approach to identifying TKI targets is through *in vitro* assays that utilise a panel of recombinant kinases to measure inhibitor binding (Lee and Wang, 2009). It is possible that imatinib and nilotinib bind other kinase target(s) which have not been detected in *in vitro* kinase screens. Even the largest kinase panels contain only a proportion of all 518 kinases of the human kinome, and the kinases not included in such screens would not be investigated for potential binding to TKIs (Hantschel et al., 2008). The pre-selection of kinase targets limits the traditional approach to kinase screening. Another limitation of these cell free screens is that they do not take into consideration *in vivo* protein interactions. Understanding the full spectrum of drug targets would enable the development of combinational therapies. Furthermore, the multi-target specificity of imatinib and nilotinib means that if their full target-spectrum is identified, the drugs could be re-purposed for other diseases.

8.5 Importance of motility-related screening for TKIs during drug development

Kinases have become one of the most important classes of proteins for drug discovery and oncology has benefited significantly from the development of small-molecule inhibitors. But acquired resistance to TKIs remains a major challenge for personalised cancer therapy. Identifying the full range of intracellular targets of a small-molecule inhibitor is extremely difficult, but vital for our understanding of toxicity, for the ability to predict how tumours may respond to particularly therapy, and for the prevention of resistance. Findings highlight that the precise molecular outcomes of imatinib and nilotinib treatment are not well defined, providing insight as to why their clinical management remains challenging.

Imatinib and nilotinib stimulate tyrosine phosphorylation of p130Cas, FAK and PXN in multiple settings, including non-transformed primary cell lines, and established and patient-derived cancer cell lines. These results highlight the necessity for screening imatinib, nilotinib and other TKIs for their effects on multiple modes of cell motility in specific cancers and other diseases where therapeutic efficacy is being investigated.

Appendix

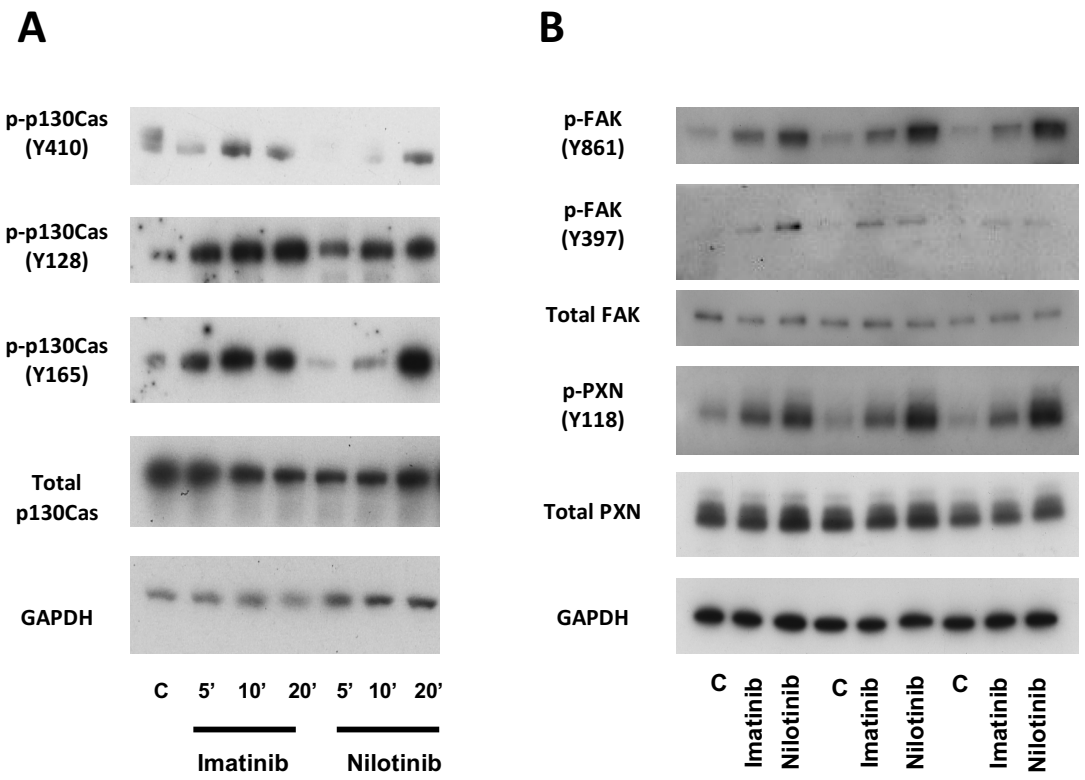


Figure A1 Imatinib and nilotinib treatment leads to increased tyrosine phosphorylation of additional p130Cas and FAK tyrosine residues. U87MG cells (~80% confluent) were incubated in SFM for ~18hr prior to treatment with **(A)** either SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 5, 10 or 20 minutes ('); (**B**) either SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies.

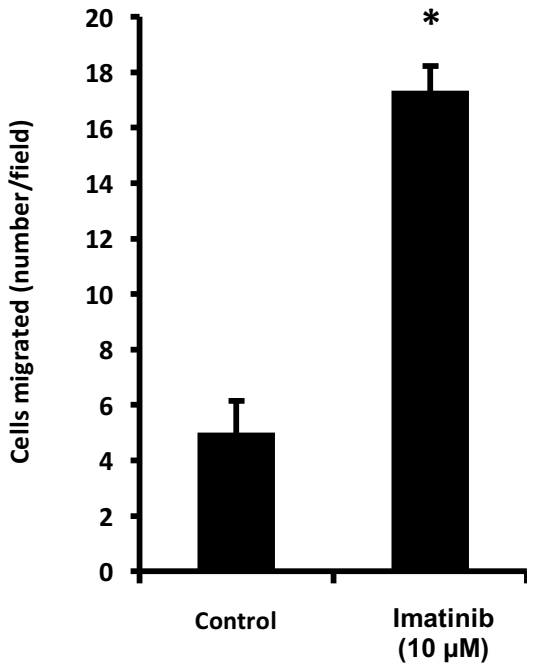


Figure A2 Imatinib and nilotinib treatment leads to increased 2D cell motility of U87MG cells. Transwell cell culture inserts were inserted into a 24-well plate. SFM supplemented with 10 μM imatinib or vehicle were placed in the bottom chamber, and U87MG glioma cells in suspension (1.5 x 10^5 cells/well) were added to the top chamber and incubated at 37˚C for 6h. Cells that had not migrated or had only adhered to the upper side of the membrane were removed before membranes were fixed and stained. Cells that had migrated to the lower side of the membrane were counted in four random fields per well. Data from three independent experiments are presented as means ± s.e.m, expressed as the number of cells migrating per field; *$p<0.01$ compared to vehicle treated control. *These experiments and quantification were performed by Dr. Paul Frankel, UCL.*

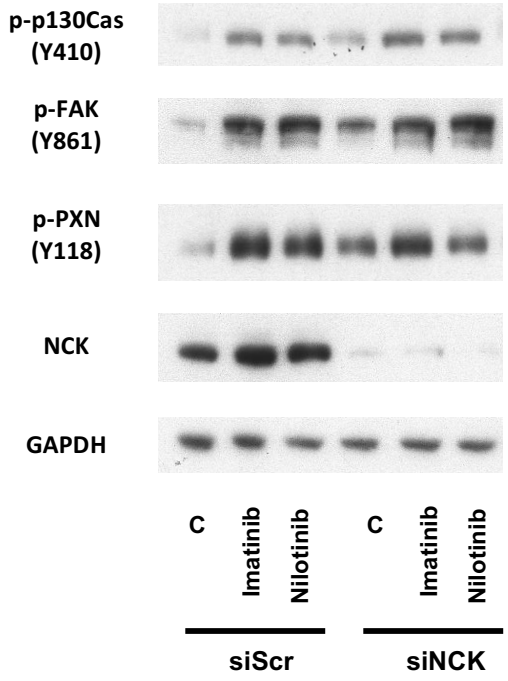


Figure A3 Imatinib- and nilotinib-induced increases in p130Cas, FAK and PXN tyrosine phosphorylation are not dependent on NCK. U87MG cells were transfected with siRNA targeting NCK (siNCK) at a concentration of 25 nM, or with 25 nM of a control scrambled siRNA (siScr). 48hr post transfection, cells were incubated in SFM for ~18hr prior to treatment with SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies.

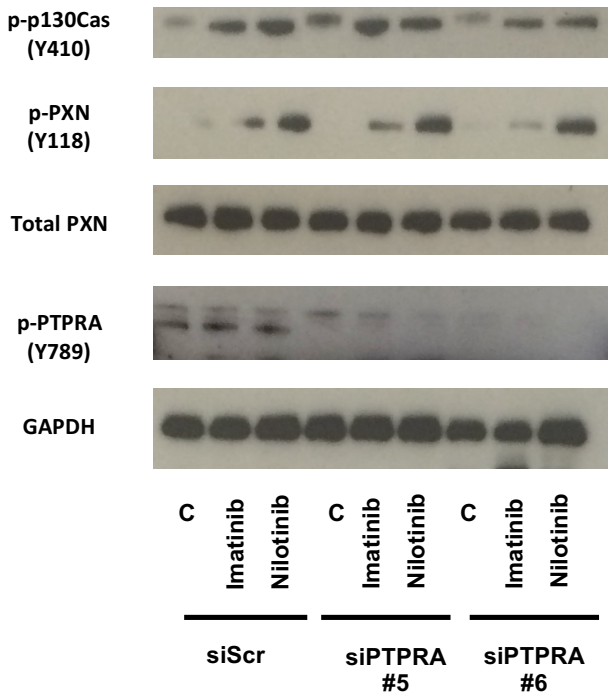


Figure A4 Silencing PTPRA does not affect imatinib and nilotinib stimulation of p130Cas and PXN tyrosine phosphorylation. U87MG cells were transfected with two different siRNAs targeting PTPRA (siPTPRA) at a concentration of 25 nM, or with 25 nM of a control scrambled siRNA (siScr). 48hr post transfection, cells were incubated in SFM for ~18hr prior to treatment with SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies.

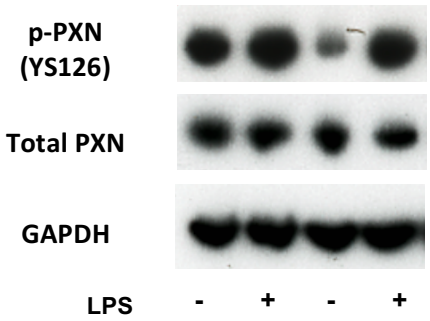


Figure A5 LPS treatment of U87MG cells. U87MG cells (80% confluent) were incubated in SFM for ~18hr prior to treatment with vehicle control (-), or 1 μg/mL LPS (+) for 60 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies.

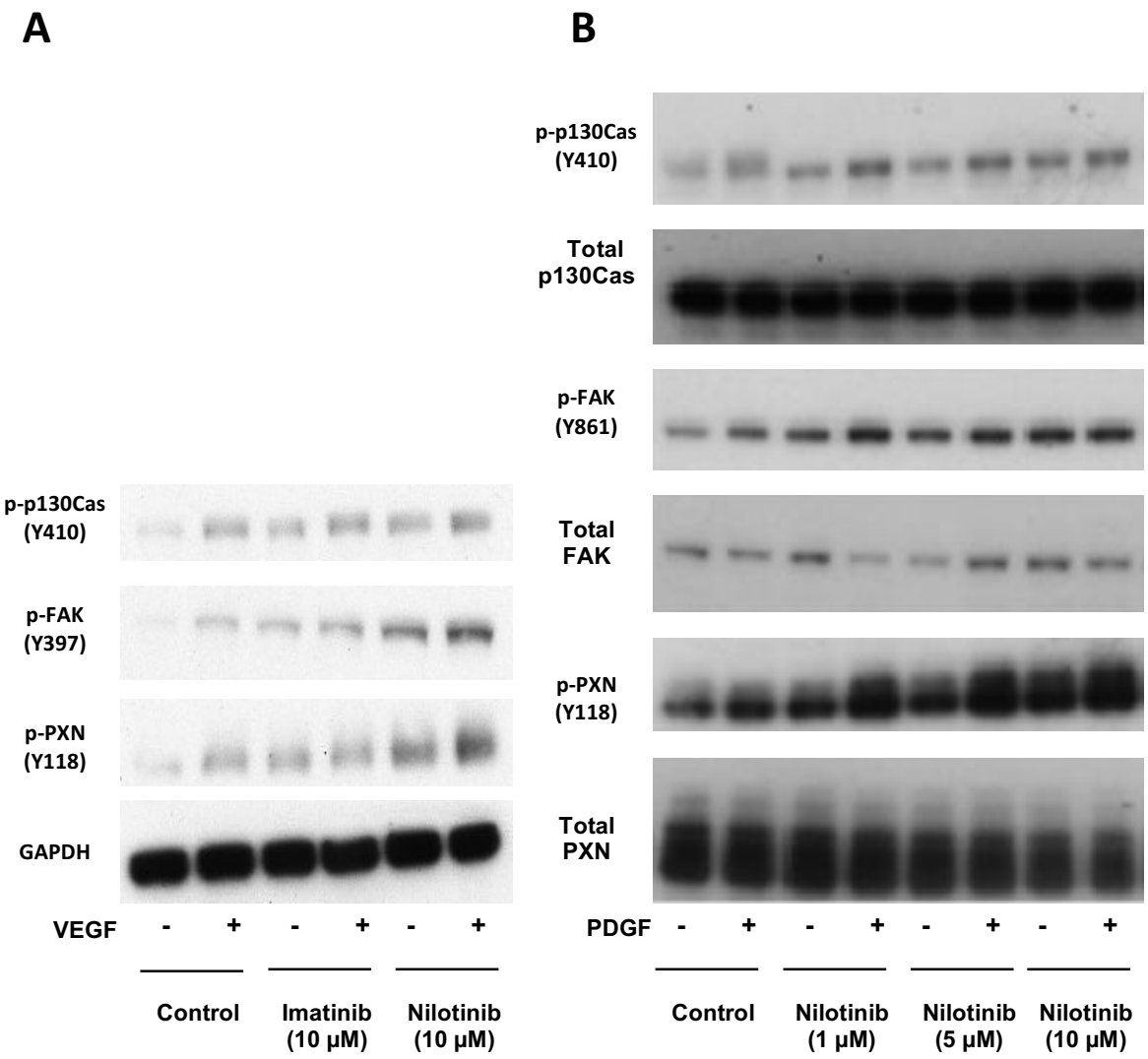


Figure A6 Imatinib and nilotinib treatment of non-cancerous cells leads to increased tyrosine phosphorylation of p130Cas, FAK and PXN. (A) HUVECs (80% confluent) were incubated in SFM for ~18hr prior to treatment with vehicle control, or 10 µM imatinib or 10 µM nilotinib for 20 minutes. Cells were then incubated for 10 minutes with 25ng/ml of VEGF (+) or vehicle control (-). Cell lysates were then prepared, blotted, and probed with the indicated antibodies. (**B**) HCASMCs were incubated in SFM for ~18hr prior to treatment with vehicle control, or different concentrations of nilotinib for 30 minutes. Cells were then incubated for 10 minutes with 50ng/ml of PDGF (+) or vehicle control (-). Cell lysates were then prepared, blotted, and probed with the indicated antibodies. *These experiments were performed by (**A**) Dr. Ian Evans, UCL; (**B**) Miss Lisa Im, UCL.*

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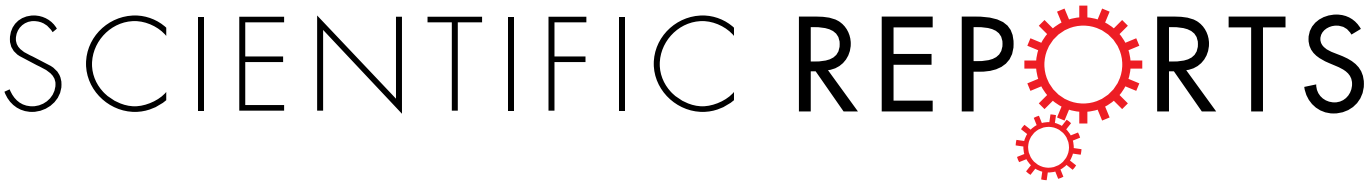

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Imatinib and Nilotinib increase glioblastoma cell invasion via Abl-independent stimulation of p130Cas and FAK signalling

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Imatinib was the first targeted tyrosine kinase inhibitor to be approved for clinical use, and remains first-line therapy for Philadelphia chromosome (Ph+)-positive chronic myelogenous leukaemia. We show that treatment of human glioblastoma multiforme (GBM) tumour cells with imatinib and the closely-related drug, nilotinib, strikingly increases tyrosine phosphorylation of p130Cas, focal adhesion kinase (FAK) and the downstream adaptor protein paxillin (PXN), resulting in enhanced cell migration and invasion. Imatinib and nilotinib-induced tyrosine phosphorylation was dependent on expression of p130Cas and FAK activity and was independent of known imatinib targets including Abl, platelet derived growth factor receptor beta (PDGFRβ) and the collagen receptor DDR1. Imatinib and nilotinib treatment increased two dimensional cell migration and three dimensional radial spheroid invasion in collagen. In addition, silencing of p130Cas and inhibition of FAK activity both strongly reduced imatinib and nilotinib stimulated invasion. Importantly, imatinib and nilotinib increased tyrosine phosphorylation of p130Cas, FAK, PXN and radial spheroid invasion in stem cell lines isolated from human glioma biopsies. These findings identify a novel mechanism of action in GBM cells for two well established front line therapies for cancer resulting in enhanced tumour cell motility.

Abnormal or dysregulated tyrosine kinase (TK) activity represents a large proportion of oncogenic activity across a broad range of cancers. TK mutation, enhanced expression and autocrine stimulation can lead to downstream signalling that is responsible for enhanced migration, proliferation, angiogenesis and survival of cancer cells[1,2].

Given their cardinal role in tumourigenesis, TKs have been the target for the development of inhibitors as therapeutics. The constitutively active oncoprotein BCR-ABL tyrosine kinase is the driver of Philadelphia chromosome (Ph+)-positive chronic myeloid leukaemia (CML)[3]. Imatinib, a BCR-ABL inhibitor (Gleevec, Novartis Pharmaceuticals Corporation, East Hanover, NJ), was the first selective tyrosine-kinase inhibitor (TKI) to be approved for the treatment of a cancer in 2002[4]. Imatinib is currently first-line therapy for Ph+-CML leading to remission in the majority of CML patients, and is also used for treatment of other malignancies including gastrointestinal stromal tumours (GIST). Imatinib was developed to bind to the ATP-binding pocket of BCR-ABL, competing with ATP, and thus blocking kinase activity[1,2]. Nilotinib, a second generation TKI, shares a very similar target spectrum with imatinib and was approved in 2010 to provide second-line treatment in case of resistance or intolerance to imatinib[5]. However, a considerable amount of CML patients do not respond favourably to nilotinib after imatinib treatment[6].

Despite encouraging clinical results for CML, and for GIST[7], imatinib has failed clinical trials for glioblastoma, where it shows no significant inhibition of tumour growth or extension of survival[8,9] Imatinib and nilotinib potently inhibit tyrosine kinases including ARG, c-KIT, PDGFR and DDR1. Moreover, imatinib and nilotinib are reported to cause activation of intracellular kinases including the PI3K, Akt and ERK pathways[3,7,10]. Inhibition of other TKs and co-activation of signalling pathways may account both for the development of imatinib resistance in Ph + CML and GIST, and imatinib's lack of efficacy in glioblastoma. The functional consequences of

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imatinib and nilotinib treatment on enhanced signalling in tumour cells remain poorly understood. In particular, their effects on cell functions modulating tumour behaviour are essential for understanding critically important aspects of drug treatment including non-responsiveness, the development of resistance, and the occurrence of side-effects.

The acquisition of enhanced cell motility provides tumour cells with the capacity to invade their surrounding tissue and metastasise, and is considered one of the "hallmarks of cancer"[11]. In this study, we demonstrate that imatinib and nilotinib treatment of glioblastoma and patient-derived glioblastoma stem cells results in increased tyrosine phosphorylation of several signalling proteins centrally important for cell motility including p130Cas, focal adhesion kinase (FAK), and paxillin (PXN), and strikingly increases tumour cell and stem cell migration and invasion. Surprisingly, these effects are independent of the known imatinib and nilotinib targets, ABL, ABL2 (ARG), c-KIT, PDGFRβ and DDR1.

Our findings point to a novel and important effect of imatinib and nilotinib upon tumour cell motility. These data may provide insight as to why imatinib has failed clinical trials for glioma, and have implications for understanding mechanisms underlying the development of imatinib and nilotinib resistance in other human malignancies.

Experimental

Cell culture. U87, U251, and U118 glioma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) foetal calf serum (FCS) supplemented with Pen/Strep (1:100; P4333-Sigma).

Derivation of Human GBM stem cell lines. All patients gave informed consent before the surgical intervention. The storage of human tissue is governed by the Human tissue Act (UK; HTA License #'s 12054). The use of tissue and cells has been approved by the National Hospital Ethics Committee (LREC 08/0077) and all methods were carried out in accordance with the approved guidelines. All tumours were diagnosed as glioblastoma (WHO grade 4) by neuropathologists. The samples were taken directly from the operating theatre and placed in cold Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12). The samples were finely minced, erythrocytes lysed by ACK buffer (Invitrogen) and tissue dissociated using Trypsin/EDTA. The resulting suspension was centrifuged and pellets re-suspended in DMEM/F12 medium supplemented with B27, bFBF, EGF and penicillin-streptomycin. Fresh medium was added to the cell suspensions every 3–5 days. When neurospheres formed, the suspension was transferred to flasks coated with laminin (Sigma). Adherent monolayer cells were sub-cultured by treatment with Trypsin/EDTA and plating them onto laminin coated plates for western blotting experiments or directly used for spheroid formation as described below.

Antibodies, reagents and small interfering RNAs (siRNAs). Antibodies to Phospho-p130Cas (Y410), DDR1, ABL1, ERK, Phospho-ERK (T202/Y204), Phospho-Paxillin (Y118), and Phospho-PDGF-R β (Y751) were from Cell Signalling Technology Inc., (Danvers, MA, USA). Antibodies to PDGFR-β, focal adhesion kinase (FAK; A-17), β1 Integrin, β3 Integrin, c-RAF, B-RAF, Paxillin (H-114), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; V-18) were from Santa Cruz Inc., (Heidelberg, Germany). Secondary antibodies to mouse, goat and rabbit were also from Santa Cruz Inc. Antibody to Phospho-FAK (Y861) was purchased from Life Technologies (Carlsbad, USA). Antibody to p130Cas antibody was from BD Transduction Laboratories (Oxford, UK). The antibody to ABL2 (N1N3) was from GeneTex (Irvine, USA). The Fak/Pyk2 inhibitor PF573228 was purchased from Tocris Bioscience (Bristol, UK). PDGF-BB was purchased from Peprotech (London, UK). Dimethyl sulphoxide (DMSO) was purchased from Sigma. U0126, Imatinib and Nilotinib were purchased from Source Bioscience (UK). The pH2B-GFP plasmid, encoding histone H2B fused to the green fluorescent protein, was obtained from Professor Sibylle Mittnacht (UCL Cancer Institute).

The following small interfering (si) RNAs were purchased from Dharmacon (GE Healthcare, UK):

si ITGB1-1: 5′-GAACAGAUCUGAUGAAUGA-3′
si ITGB1-2: 5′-CAAGAGAGCUGAAGACUAU-3′
si ITGB3-1: 5′-CUCUCCUGAUGUAGCACUUAA-3′
si ITGB3-2: 5′-CACGUGUGGCCUGUUCUUCUA-3′
si p130Cas #2: 5′-GGUCGACAGUGGUGUGUAU-3′

The following siRNA was purchased from Life Technologies (Carlsbad, USA):
si p130Cas #1: 5′ -GAGUUUGAGAAGACCCGATT-3′

The following siRNAs were purchased from Qiagen (Crawley, UK):
AllStars Negative Control

siPDGFR-β 5′-GGAACGTGCTCATCTGTGA-3′
siABL1 (#10) 5′-ACGCACGGACATCACCATGAA-3′
siABL2 (#8) 5′-AACCCTGTCCTTAATAACTTA-3′
siCRAF (#5) 5′-AAGACGTTCCTGAAGCTTGCC-3′
siBRAF (#1) 5′-AACATATAGAGGCCCTATTGG-3′
siDDR1 (#9) 5′-ACGGTGTGAATCACACATCCA-3′

siRNA Transfection. U87 and U251 glioma cells at 60% confluence were transfected with Lipofectamine 2000 (Invitrogen) using 25 nM final concentration of siRNA as described[12].

Immunoblotting. For immunoblotting, cells were lysed in a solution containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, complete protease inhibitor (Roche) and phosphatase inhibitors I & II (Sigma) and analysed by SDS-PAGE using 4 to 12% Bis-Tris gels (NuPAGE; Invitrogen), followed by electro-transfer onto Invitrolon polyvinylidene difluoride membranes (Invitrogen). Membranes were blocked with 5% (wt/vol) non-fat dry milk and 0.1% (vol/vol) Tween-20 in Tris-buffered saline for 1 hour at room temperature, before being probed with the primary antibody by overnight incubation at 4 °C, followed by incubation for 1 hour at room temperature with a horseradish peroxidase-linked secondary antibody (Santa-Cruz) and detection using ECL reagents (Bio-Rad, Hercules, USA), following the manufacturer's protocol. Immunoblots were quantified by scanning of films with a calibration strip and analysis by densitometry using Image J (US National Institutes of Health; http://rsb.info.nih.gov/ij).

Immunofluorescent staining and Confocal Imaging. For immunofluorescent staining, cells were fixed in 4% paraformaldehyde in PBS for 60 min followed by permeabilisation in 0.2% Triton X100 for 30 min. Antibody incubations were performed overnight at 4 °C in 1% BSA, 0.1% Tween20 in PBS. Confocal imaging was performed using a LEICA SPE2 upright microscope running LEICA-LAS software using sequential imaging capture.

Transwell chemotactic migration assay. This assay was performed as described previously[12]. Briefly, Transwell cell culture inserts (Falcon; BD Biosciences, Oxford, UK), were inserted into a 24-well plate. Serum free media supplemented with or without imatinib or vehicle were placed in the bottom chamber, and U87 glioma cells in suspension (1.5×10^5 cells/well in serum free DMEM) were added to the top chamber and incubated at 37 °C in for 6 h. Cells that had not migrated or had only adhered to the upper side of the membrane were removed before membranes were fixed and stained with a Reastain Quik-Diff kit (IBG Immucor Ltd, West Sussex, UK). Cells that had migrated to the lower side of the membrane were counted in four random fields per well at 20× magnification using an eyepiece indexed graticule.

Cell Proliferation assay. Proliferation of U87MG cells (stably expressing H2B-GFP) was determined in 96-well plates (seeding density of 2000 cells per well) by assessing total cell fluorescence intensity per well in living cells using an IncuCyte Zoom (Essen Bioscience) for up to 96 hours.

Three dimensional (3D) spheroid invasion assay. Spheroids were generated using the metho-cellulose technique as previously described[13,14]. siRNA transfection were carried out on cells as described above. Following 24 hours transfection, cells were trypsinised, and 5×10^4 cells/ml were suspended in a medium containing a 4:1 (v/v) mixture of 10% FCS in DMEM and methylcellulose. Spheroids were produced by pipetting 100 μl of the cell suspension into a well of a 96-well round bottomed non-tissue culture plate and incubating for 24 hours (37 °C, 5% CO_2). Spheroids were collected and embedded in Collagen I plugs (2.1 mg/ml) prepared from fibrillar bovine collagen I (3.1 mg/ml; PureCol) by dilution in DMEM in accordance with the manufacturer's protocol (Nutacon, The Netherlands). The collagen I solution was supplemented with either DMSO or 10 uM imatinib or 1 uM nilotinib. Spheroids were allowed to invade for 48 hours followed by fixation in 4% formaldehyde. Spheriod Invasion was determined by measuring the circular area of the spheroid core and the rim of Invasion using Image J. The rim of invasion was determined by the circular distance from the edge of the core to the edge of contiguous invading cells[13,15].

Scratch wound assay. Cells were seeded to confluence, scratched evenly at the centre, and treated with imatinib, nilotinib, or vehicle control. Rate of wound closure was measured using an Incucyte Zoom (Essen Bioscience, UK). Images were captured every hour for 30 hours.

Immunoprecipitation. Cells were washed with PBS, lysed in NP40 (50 mM Tris–HCl at pH 8, 150 mM NaCl, 0.5% NP40) containing protease and phosphatase inhibitors and centrifuged for 15min at high speed (16000 *g* at 4 °Cmin). Immune complexes were collected when 1mg of cell lysate was immunoprecipitated with 2 μg of antibody or with control IgG. Lysis buffer was used to wash the beads three times before a final wash using 0.6M lithium chloride was performed. These samples or 20 μg lysate was then supplemented with sample buffer (Tris at pH 6.8, 20% glycerol, 5% SDS, β-mercaptoethanol and bromo-phenol blue), separated by SDS–PAGE, transferred to a nitrocellulose membrane and then immunoblotted.

Statistical analysis. The data displayed on graphs are means, with error bars representing the standard error of the mean (SEM). Statistical analysis was performed by two-way analysis of variance (ANOVA), or T-test where appropriate. $P < 0.05$ was considered significant.

Results

We treated the human glioblastoma multiforme (GBM) cell line U87MG with either imatinib or nilotinib and investigated their effects on key signalling components required for tumour cell motility and invasion. Tyrosine phosphorylation of p130Cas at Tyr410, FAK at Tyr861 and PXN at Tyr118 play important roles in cell migration and invasion and were used as readouts of activation of these signalling pathways[12,16,17]. Treatment with 10 μM imatinib or nilotinib caused a striking increase in tyrosine phosphorylation of p130Cas, Focal Adhesion Kinase (FAK) and Paxillin (PXN) (Fig. 1A). These effects were dose-dependent, with a significant increase detected at 1 μM and a maximal response at 10 μM for both imatinib and nilotinib in U87MG cells (Fig. 1B). Because the peak plasma/serum concentrations of imatinib and nilotinib, are approximately 5 μM and 4 μM, respectively[18,19], our data indicate that these effects occur at clinically relevant concentrations. The effects of imatinib and nilotinib on

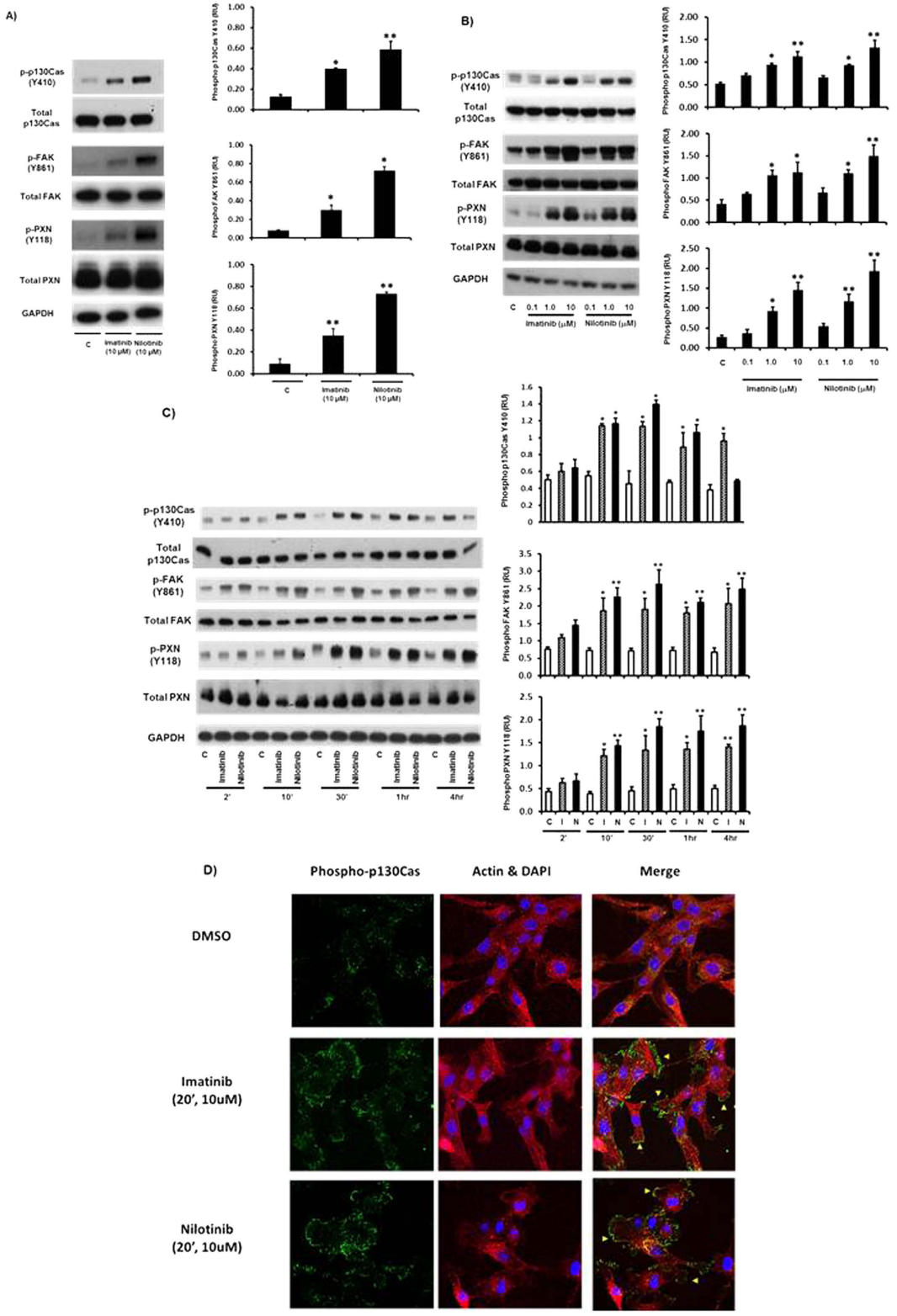

Figure 1. Imatinib and nilotinib treatment of human GBM cells leads to increased p130Cas, Focal Adhesion Kinase (FAK) and Paxillin (PXN) tyrosine phosphorylation. Cells (~80% confluent) were incubated in SFM for ~18 hr prior to treatment with either SFM & DMSO vehicle control (C), or imatinib or nilotinib at the indicated concentrations and times. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Blots shown here and in all subsequent figures unless indicated are representative of at least three separate experiments. (**A**), U87MG cells were treated with vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. (**B**), Dose dependency of imatinib or nilotinib treatment for 20 minutes in U87MG cells. (**C**), Time course of U87MG cells treated with vehicle control (C), or 10 μM imatinib or 10 μM nilotinib. Quantitation of tyrosine phosphorylation was performed by densitometry using Image J. In each panel, data from at least

three independent experiments are presented as phosphorylation relative units (RU) (means +/− s.e.m.) normalized to total protein levels; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to vehicle control (**C**). $^{\#}p > 0.05$ compared to vehicle control (C). (**D**), U87MG cells were seeded on glass cover slips and incubated in SFM for ~18 h prior to for 20 min with vehicle control (C), or 10 μM imatinib or 10 μM nilotinib. Confocal imaging was performed as described in Materials and Methods, with phosphorylated p130Cas (Y410) staining in green, actin in Red and DAPI in purple. Images are representative of at least three separate experiments. Arrows point to areas of increased p130Cas (pY410) localisation to the membrane upon treatment.

tyrosine phosphorylation of p130Cas, FAK and PXN were also rapid and sustained with a significant increase observed at 10 min, reaching a maximum at 30 min, which was maintained for up to 1 hour (Fig. 1C). After 4 hours, p130Cas tyrosine phosphorylation declined to near basal level, whereas FAK and PXN tyrosine phosphorylation remained strongly elevated. These results were further supported by the finding that imatinib and nilotinib induced p130Cas, FAK and PXN tyrosine phosphorylation in the glioma cell line, U251MG (Fig. S1).

We also examined the effect of imatinib and nilotinib treatment on the localisation of phosphorylated p130Cas in U87MG cells by performing immunofluorescence confocal microscopy. As expected the levels of phosphorylated p130Cas (Tyr 410) increased in both imatinib and nilotinib treated cells after 20 min. We also observed a striking redistribution of phoso-p130Cas to the cell membrane, localising along what appear to be membrane ruffles (Fig. 1D).

Imatinib and nilotinib are both inhibitors of the Abl family tyrosine kinases, ABL1 and ABL2 (also called ARG). Furthermore, since Abl has been reported to regulate p130Cas tyrosine phosphorylation[20], we reasoned that ABL1 and/or ABL2 could be mediating the effects of imatinib and nilotinib treatment in these glioma cell lines. We treated U87MG cells with siRNA to ABL1 and ABL2 either individually or in combination in the presence of imatinib, nilotinib or vehicle control. Knockdown of ABL1 or ABL2 alone or together had no effect on p130Cas, FAK and PXN tyrosine phosphorylation in control treated cells. Furthermore ABL1 and ABL2 silencing had no effect on imatinib and nilotinib mediated increases in p130Cas and PXN tyrosine phosphorylation, whereas tyrosine phosphorylation of FAK was reduced. Interestingly, knockdown of ABL1 and to a lesser extent ABL2 caused a decrease in total p130Cas and total PXN expression, whilst total FAK levels were unaffected (Fig. 2A). Imatinib and nilotinib inhibit additional tyrosine kinases, including platelet derived growth factor receptor beta (PDGFRβ), stem cell growth factor receptor (c-KIT), and discoidin domain receptor tyrosine kinase 1 (DDR1). However, silencing of PDGFRβ and DDR1 had no effect on increased tyrosine phosphorylation in response to imatinib and nilotinib (Fig. 2B & S2A). We were unable to detect c-KIT in U87MG cells, whereas we could readily detect c-Kit in human coronary artery smooth muscle cells (HCASMCs), indicating that c-Kit is not significantly expressed in U87MG cells (Fig. S2B).

The SRC tyrosine kinase is well known to play a role in the regulation of p130Cas, FAK and PXN tyrosine phosphorylation[20]. We therefore looked at the effect of using the SRC kinase inhibitor, PP2, in imatinib and nilotinib treated U87MG cells. We found that PP2 treatment leads to a complete abrogation of both basal and imatinib/nilotinib induced tyrosine phosphorylation of p130Cas, FAK and PXN (Fig. S3). This result led us to look at the levels of tyrosine 416 phosphorylation in SRC. We found that imatinib and nilotinib treatment did not result in any changes in the levels of Y416 phosphorylation (data not shown).

It has recently been reported that imatinib and nilotinib treatment leads to activation of MEK and ERK in several human tumour cell lines[3], and ERK signalling has been implicated in meditating signalling pathways required for migration of glioma cells[21,22]. We therefore examined the effect of imatinib and nilotinib treatment on ERK activation in U87MG cells. Although imatinib treatment increased levels of phosphorylated ERK, nilotinib treatment had no effect (Fig. S4A). Furthermore, whereas the MEK inhibitor, U0126, completely abolished levels of phosphorylated ERK in all samples treated, it had no effect on imatinib and nilotinib stimulation of p130Cas, FAK, and PXN tyrosine phosphorylation (Fig. S4A). It was recently reported that imatinib and nilotinib can bind to B-RAF and C-RAF leading to the formation of RAF hetero- and homo-dimers, and stimulate paradoxical activation of BRAF and CRAF in the presence of activated RAS[3]. We therefore considered the possibility of a RAF-dependent, MEK/ERK-independent pathway leading to increased tyrosine phosphorylation of p130Cas, FAK and PXN. However, silencing of B-RAF or C-RAF either alone or together had no effect on imatinib and nilotinib stimulated increases in tyrosine phosphorylation (Fig. S4B). Because imatinib treatment was able to increase levels of ERK phosphorylation (Fig. S4A) we looked at the effect of imatinib and nilotinib treatment on U87MG cell proliferation. As shown in Fig. S7, drug treatment has no significant effect on cell proliferation.

Integrins are known to mediate p130Cas and FAK tyrosine phosphorylation through beta 1 & beta 3 subunits[20,23]. To test the possibility that imatinib and nilotinib stimulated tyrosine phosphorylation of p130Cas, FAK and PXN occurs via an integrin signalling pathway, we treated U87MG cells with siRNA to either beta 1 or beta 3 integrins. Knockdown resulted in a marked reduction in integrin protein expression, yet had no effect on increased p130Cas, FAK and PXN tyrosine phosphorylation (Fig. S4C,D).

Because p130Cas and FAK have been reported to exist in multi-protein complexes required for cell motility[20], we examined the association of these molecules in glioma cells, and their interdependence in the response of U87MG cells to imatinib and nilotinib. Immunoprecipitation of p130Cas and subsequent immunoblotting showed that FAK and PXN were both constitutively complexed with p130Cas in U87MG glioma cells, and that these complexes were not affected by imatinib or nilotinib treatment (Fig. S5). However, targeted knockdown of p130Cas with two different siRNA significantly reduced tyrosine phosphorylation of FAK and PXN induced by imatinib and nilotinib (Fig. 3A). Furthermore, treatment of U87MG cells with the FAK inhibitor PF-573,228 (PF-228) significantly reduced the imatinib and nilotinib stimulated increases in p130Cas and PXN tyrosine phosphorylation (Fig. 3B).

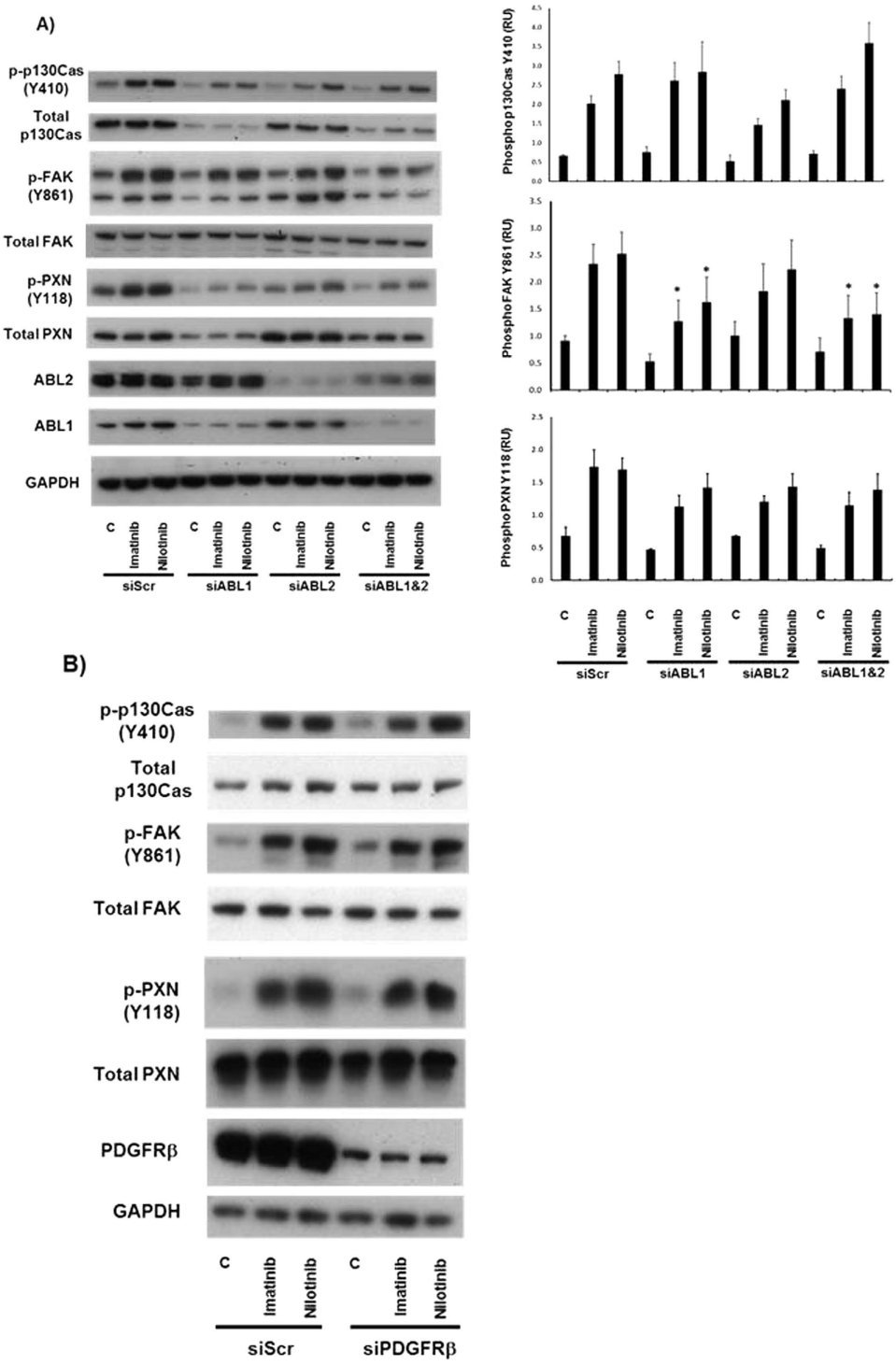

Figure 2. Imatinib and Nilotinib target proteins are not required for increased p130Cas, FAK and PXN tyrosine phosphorylation. (**A**), U87MG cells were transfected with siRNA targeting ABL1 (siABL1), ABL2 (siABL2) or together at a concentration of 25 nM, or with 25 nM of a control scrambled siRNA (siScr). (**B**) U87MG cells were transfected with siRNA targeting PDGFR Beta (siPDGFRB), cells were transfected at a concentration of 25 nM, or with 25 nM of a control scrambled siRNA (siScr). 48 hr post transfection, cells were incubated in serum-free medium (SFM) for ~18 hr prior to treatment with SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies.

Signalling via p130Cas and FAK pathways plays crucial roles in the regulation of cellular motility[13,20,24]. We therefore investigated the effects of imatinib and nilotinib treatment on glioma cell motility using both a three dimensional (3D) spheroid assay and two dimensional (2D) assays of chemotaxis and wound healing. We

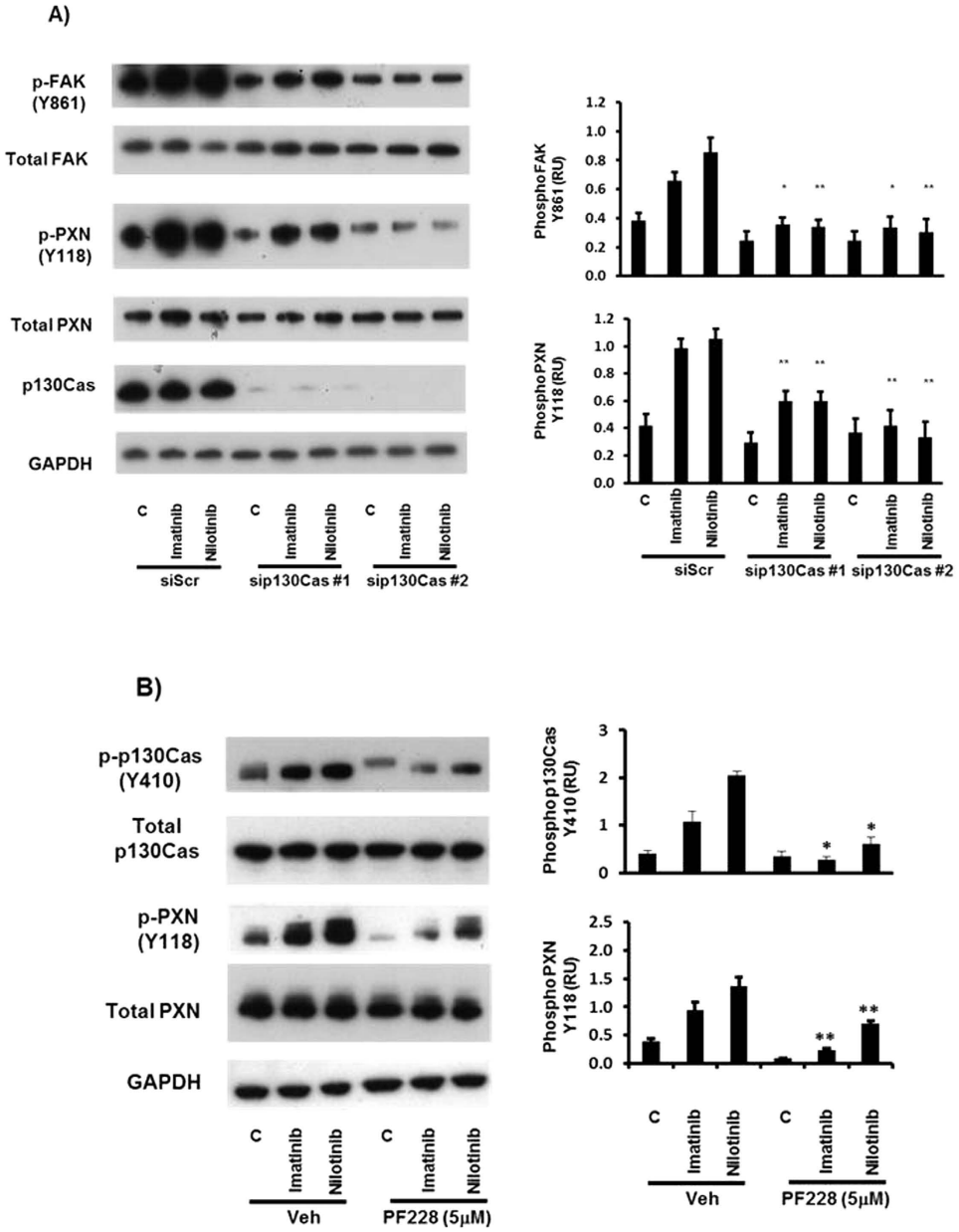

Figure 3. Increased p130Cas, FAK and Paxillin tyrosine phosphorylation is dependent on p130Cas expression and FAK kinase activity (**A**) U87MG cells were transfected with siRNA targeting p130Cas (si p130Cas) at a concentration of 25 nM, or with 25 nM of a control scrambled siRNA (siScr). 48 hr post transfection, cells were incubated in serum-free medium (SFM) for ~18 hr prior to treatment with SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. (**B**) U87MG cells (~80% confluent) were incubated in SFM for ~18 hr prior to pre-incubation for 30 min with 5 μM PF573228 or the vehicle (0.05% DMSO) (C) prior to treatment with SFM & DMSO vehicle control (C), or 10 μM imatinib or 10 μM nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Quantitation of tyrosine phosphorylation was performed by densitometry using Image J. In each panel, data from at least three independent experiments are presented as phosphorylation relative units (RU) (means +/− s.e.m.) normalized to total protein levels; *$p < 0.05$ compared to vehicle control (C).

generated spheroids from U87MG, U251 and U118MG GBM cell lines and embedded them in collagen I plugs supplemented with either serum free medium (SFM) and DMSO (Veh), or SFM containing imatinib or nilotinib. Treatment with either imatinib or nilotinib alone for 48 hours resulted in a striking increase in radial invasion compared to the vehicle control spheroids (Fig. 4A–C). In transwell assays of chemotactic cell motility, we found surprisingly that imatinib acted as a chemo-attractant agent and was able to stimulate increased migration in U87MG cells (Fig. S6A). Furthermore, in wound healing assays in U251MG cells we found that either imatinib or nilotinib treatment resulted in increased rates of wound closure compared to control vehicle-treated cells (Fig. S6B). We next investigated the role of p130Cas, FAK and the MEK/ERK pathway in imatinib and nilotinib

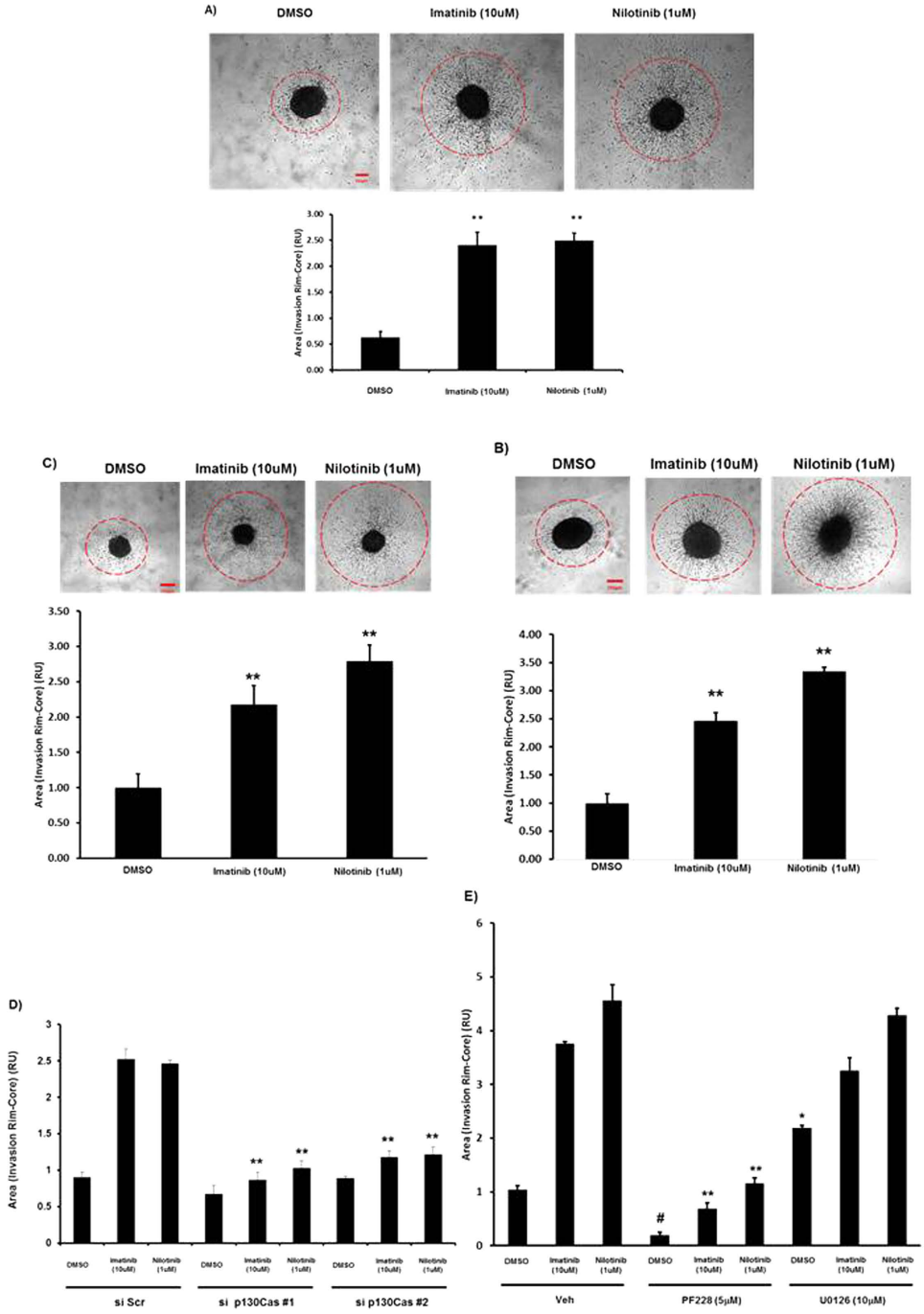

Figure 4. Imatinib and Nilotinib treatment of human GBM cells leads to increased 3D radial invasion of GBM cell spheroids (**A**) U87MG. (**B**) U251MG. (**C**) U118MG. (**A**–**C**) Equal amounts of cells were used to generate spheroids as described in Materials and Methods. 24 hours after spheroid production, spheroids were imbedded in a collagen gel and incubated in either SFM & DMSO vehicle control or 10 μM imatinib or 1 μM nilotinib for an additional 48 hours. $^{**}p < 0.01$ compared to SFM & DMSO vehicle control. (**D**) Spheroids derived from U87MG cells treated with siRNA to p130Cas (si p130Cas #1 & #2) or control scrambled siRNA (siScr) were imbedded in collagen containing either SFM & DMSO vehicle control or 10 μM imatinib or 1 μM nilotinib and treated as above. $^{**}p < 0.01$ compared to imatinib and nilotinib siScr respectively.(**E**) Spheroids derived from U87MG cells were imbedded in collagen containing either SFM & DMSO vehicle control, 5 μM PF573228 or 10 μM U0126 and treated as above. $^{**}p < 0.01$ compared to imatinib and nilotinib DMSO vehicle control respectively. $^{\#}p > 0.05$ compared to SFM & DMSO vehicle control. Spheroids were fixed in 4% PFA and invasion was determined by measuring the area corresponding to the invasion rim minus the area of the core for at least 3 different spheroids per condition. Data from three independent experiments are presented as relative area units (RU) (means +/− s.e.m.).

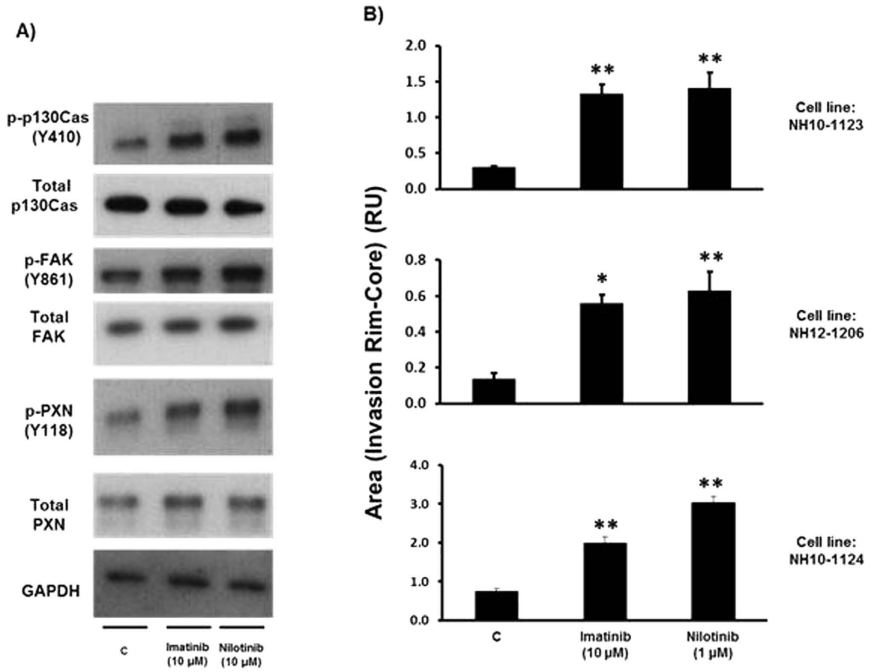

Figure 5. Imatinib and Nilotinib treatment of stem cells from human GBM biopsies leads to increased p130Cas, Focal Adhesion Kinase (FAK) and PXN tyrosine phosphorylation and increased 3D spheroid radial invasion. (**A**) Stem cells established from a human GBM biopsy (cell line: NH10-1124) (~80% confluent) were incubated in SFM for ~18 hr prior to treatment with either SFM & DMSO vehicle control (C), or imatinib or nilotinib for 20 minutes. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. (**B**) Spheroids derived from the indicated stem cell lines established from human GBM biopsies were imbedded in a collagen gel and incubated in either SFM & DMSO vehicle control (C), or 10 μM imatinib or 1 μM nilotinib for an additional 48 hours. Spheroids were fixed in 4% PFA and invasion was determined by measuring the area corresponding to the invasion rim minus the area of the core for at least 3 different spheroids per condition. Data from three independent experiments are presented as relative area units (RU) (means +/− s.e.m.). $^{*}p < 0.05$, $^{**}p < 0.01$ compared to SFM & DMSO vehicle control (C).

stimulated spheroid invasion. Spheroids from U87MG cells treated either with siRNA to p130Cas or with pharmacological inhibitors of FAK (PF-228) or MEK (U0126) were prepared and then treated with imatinib and nilotinib. Silencing of p130Cas, or treatment with PF-228 significantly reduced invasion induced by imatinib and nilotinib (Fig. 4D,E), whereas U0126 treatment had no significant effect on radial invasion in imatinib and nilotinib treated spheroids, although U0126 alone resulted in a small but significant increase in radial invasion compared with SFM vehicle control (Fig. 4E). The importance of cancer stem cells (CSCs) in tumour progression and their role in driving tumour relapse after treatment is emerging rapidly. GBM is one of the first tumours where (CSCs) were identified. Glioma stem cells (GSCs) are thought to be responsible for tumour maintenance and critically important in recurrence after resection[25]. We therefore examined the relevance of our findings in glioma cell lines to GSCs by investigating the effects of imatinib and nilotinib on GSCs isolated from human GBM biopsies. Imatinib and nilotinib treatment of a recently isolated GSC line significantly increased tyrosine phosphorylation of p130Cas, FAK and PXN (Fig. 5A). Furthermore, imatinib and nilotinib treatment of spheroids generated from the GSC line and two additional GSC lines significantly increased 3D radial invasion compared to the vehicle control (Fig. 5B).

Discussion

Here we show that treatment of human glioma cell lines with the tyrosine kinase inhibitors imatinib and nilotinib produces a rapid and striking increase in tyrosine phosphorylation of p130Cas, FAK and PXN, key signalling molecules required for cell motility[20,24]. These effects were induced at concentrations similar to clinically relevant drug concentrations. The finding that imatinib and nilotinib treatment stimulates tyrosine phosphorylation as early as 10 minutes indicates that this is an immediate response though a direct drug effect on signalling components distal to p130Cas and FAK pathways. This is in contrast to the indirect result of longer-term effects on metabolism or gene expression[3,26,27]. Furthermore, localisation of tyrosine phosphorylated p130cas to the cell membrane in imatinib and nilotinib treated cells is in agreement with the previously reported roles for p130Cas in cell motility[20].

A surprising result of this study is that the effects of imatinib and nilotinib on p130Cas, FAK and PXN tyrosine phosphorylation were independent of the major known tyrosine kinase targets for these drugs. Knockdown of ABL1, ABL2 or both ABL1 & ABL2 had no effect on imatinib and nilotinib stimulated tyrosine phosphorylation of p130Cas and PXN. We did however detect a significant reduction in imatinib and nilotinib stimulated tyrosine

phosphorylation of FAK in cells treated with siRNA to ABL1, and both ABL1 & ABL2. This is most likely due to the reduction in total p130Cas expression observed in ABL1 and ABL1 & ABL2 treated cells. This is supported by our results showing that small changes in p130Cas knockdown efficiency between two different siRNAs exhibit differences in the reduced levels of imatinib and nilotinib stimulated tyrosine phosphorylation of FAK and Paxillin. Furthermore, knockdown of PDGFRβ, and DDR1 had no direct effect on imatinib and nilotinib stimulated tyrosine phosphorylation of p130Cas, FAK and PXN. Whilst the lack of expression of c-Kit in U87MG cells indicates it is not responsible for these effects. It is important to note that these experiments also preclude the possibility of kinase independent roles for these targets. SRC is the main kinase responsible for tyrosine phosphorylation of these proteins[20]. We found that pharmacological inhibition of SRC leads to a complete abrogation of both basal and imatinib/nilotinib induced tyrosine phosphorylation of p130Cas, FAK and PXN. However, imatinib and nilotinib treatment did not result in any changes in the levels of tyrosine 416 phosphorylation in SRC, making it difficult to interpret these data in the context of a direct role for SRC.

Another potential mechanism underlying the effects of imatinib and nilotinib on motility-associated tyrosine phosphorylation is increased signalling through the RAF/MEK/ERK pathway, which has recently been reported to occur in several human tumour cell lines in response to these drugs[3]. Whilst we observed increased ERK phosphorylation in response to imatinib treatment, nilotinib treatment did not lead to increased ERK phosphorylation, possibly pointing to engagement of different signalling pathways in glioma cells. Furthermore, inhibition of MEK/ERK signalling using U0126 had no effect on imatinib- and nilotinib-stimulated tyrosine phosphorylation. In addition, we observed no effect of silencing of B-RAF or C-RAF, either alone or together, on increased tyrosine phosphorylation in response to imatinib and nilotinib.

Many studies have shown a role for integrin receptors and integrin signalling in the regulation of p130Cas, FAK and PXN tyrosine phosphorylation either through "inside-out" or "outside-in" signalling[20,24]. We however, found no effect on increased tyrosine phosphorylation in cells which were depleted of integrin β1 or Integrin β3 by gene silencing. This result is in agreement with our recent work showing that p130Cas tyrosine phosphorylation and 3D invasion were independent of integrin β1 in U87MG cells[28].

Both p130Cas and FAK have been shown to signal through multi-protein complexes, required for cell motility[13,20], yet the individual contributions of these molecules in such complexes is not completely understood. Our previous studies show that platelet derived growth factor (PDGF-BB) or hepatocyte growth factor (HGF) stimulated tyrosine phosphorylation of p130Cas in U87MG cells was independent of FAK kinase activity[12]. However, we show here that both p130Cas expression and FAK kinase activity are required for the increased tyrosine phosphorylation of p130Cas, FAK, and PXN, and glioma cell spheroid invasion induced by imatinib and nilotinib. This supports the conclusion that imatinib and nilotinib impact upon p130Cas and FAK complexes via a signalling pathway distinct from that mediating PDGFRβ and c-Met signalling and motility in U87MG cells[12].

An important finding of this study is that, consistent with increased tyrosine phosphorylation of p130Cas, FAK, and PXN induced by imatinib and nilotinib treatment, these drugs strikingly increased motility in 2D and 3D models using multiple human glioma cell lines. The conclusion that the pro-invasive activity of imatinib and nilotinib is mediated through p130Cas and FAK signalling is supported by the finding that the response to these drugs was strongly reduced by silencing of p130Cas and inhibition of FAK, but not by the inhibition of MEK.

Imatinib and nilotinib are successfully used in the clinic as front line therapies for Ph+ CML. In this context, Ph+ CML is the result of a single driver mutation and does not possess the genetic heterogeneity that is observed in GBM[29]. CML is a cancer of the white blood cells, resulting in increased and unregulated growth of predominantly myeloid cells in the bone marrow and the accumulation of these cells in the blood[30]. These cells are non-adherent and do not rely on adherent modes of cell motility for tumour progression. Imatinib-resistant Ph+ CML patients are either initially resistant (primary) or develop resistance over the course of treatment (acquired)[31]. Patients with advanced stage disease frequently have both[32]. The process involved in both primary and acquired imatinib-resistance can be divided into either BCR-ABL-dependent or BCR-ABL -independent mechanisms. BCR-ABL-dependent mechanisms include point mutations within the kinase domain resulting in reduced drug potency, while BCR-ABL -independent mechanisms are more varied and poorly understood. However, there are reports of imatinib-resistant Ph+ CML tumour cells undergoing a mesenchymal-like conversion associated with increased levels of FAK tyrosine phosphorylation and increased adherence and invasiveness[26]. Furthermore, imatinib is used as an adjuvant therapy in patients with Gastrointestinal stromal tumour (GIST) and there are reports of imatinib treated GIST cells showing increased FAK tyrosine phosphorylation after long term treatment (6–24 hours)[27,33]. On the basis of our results, we also propose that pharmacological modulation of FAK activity could provide an approach to boost imatinib and nilotinib efficacy and limit resistance to these drugs in CML & GIST and other cancers in which imatinib is used, a possibility that warrants further investigation. In this context, it is of interest that pharmacological inhibition of FAK using TAE226 can synergise with nilotinib in reducing Ph+ CML growth[34,35]. Furthermore, a FAK-selective inhibitor (TAG372) induced apoptosis of imatinib-resistant GIST-T1 cells and decreased the imatinib IC50[33].

The results presented here suggest a potential adverse effect of the use of imatinib and nilotinib treatment in GBM tumours, which is dependent on augmented adherent tumour cell motility. The relevance of our study for human GBM is further underscored by the finding that imatinib and nilotinib treatment of stem cell lines derived from human GBM biopsies also increased p130Cas, FAK and PXN tyrosine phosphorylation and radial invasion of spheroids generated from these cell lines. Imatinib and nilotinib treatment resulting in enhanced invasion through increased p130Cas and FAK signalling could lead to selection of tumour cells with increased motility independent of c-ABL and PDGFRβ signalling pathways. Recently, imatinib was tested in a multi-centre phase III clinical trial for patients with recurrent GBM. The results indicated that there were no clinically meaningful differences between mono therapies (Hydroxy Urea or Temozolimide) or combination therapies with imatinib[36] and the primary study end point was not met. GBM is an extremely aggressive cancer due in part to its highly invasive nature. Our findings indicate important and unforeseen adverse effects of imatinib and nilotinib treatment on

tumour cell motility, which could be a significant contributor to the lack of clinical efficacy observed in these trials. These results point to the necessity for screening imatinib, nilotinib and other members of this class of TKI for their effects on multiple modes of cell motility in specific cancers and other diseases where therapeutic efficacy is being investigated.

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Author Contributions

A.F., I.M.E. and P.F. designed the study, performed the experiments, analysed the data and wrote the paper. K.P., N.L. and A.B. performed the experiments. I.C.Z. designed the study, analysed the data and wrote the paper. N.L., K.S. and S.B. generated essential reagents and cell lines.

Additional Information

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